Microbial biodegradation potential of hydrocarbons evaluated by colorimetric technique: a case study

E. D. Bidoia, R. N. Montagnolli and P. R. M. Lopes
Departamento de Bioquímica e Microbiologia, Instituto de Biociências, UNESP – Univ Estadual Paulista, Av. 24A, 1515, 13506-900 Rio Claro-SP, Brazil

The increasing industrial development promotes serious environmental damage due to pollution of the environment. Regarding the petrochemical industry, contamination by oil and its derivatives causes the degradation of terrestrial and aquatic ecosystems. Thus, control and treatment strategies to combat the hazardous effects of oil pollution are needed. However, conventional physical-chemical treatments have high costs and can generate residues that are toxic to the biota. Allying high efficiency and low cost, bioremediation processes represent an extremely important way of recovering contaminated areas among several other cleaning-up techniques. These strategies involve microorganisms and their metabolism in biodegrading organic compounds. Also, the use of nutrients, aeration, pH and temperature adjustments or the addition of substances could make the biodegradation process easier. In order to accomplish this, screening and evaluation methods adapted to a potentially biodegrading microbiota in different types of contaminants have been established. Viable methods in biodegradation data generation during biotechnological process application are fundamental in the elaboration of original references about the biodegradability of certain substances. There are many techniques capable of precisely evaluating biodegradation processes, including colorimetric methods. The isolation, characterization and profile of specific bacteria in petrol derived oil biodegradation capacity studies are important when deciding the correct bioremediation strategy. Different microorganism species have different biodegradation capabilities. Due to this fact, the elaboration of different types of oil biodegradation profiles by different bacteria is an important task for selecting microorganisms in bioremediation processes. In order to accomplish this, screening and evaluation methods adapted to potentially biodegrading bacteria have been established. One of these methods adapted to biodegradation evaluation is colorimetry, which is a technique used to evaluate the biodegradation of some substances. DCPIP based colorimetric technique provides enough data on hydrocarbons used as metabolic substrates by microorganisms. The concentration detection is possible due to the absorbance determination in a specified light specter. The 2,6-dichlorophenol indophenol (DCPIP, redox potential +0.217 V) indicator is widely used in colorimetric processes. Its property is the color change from blue to transparent when subjected to chemical reduction. The indicator, when oxidized is blue and when reduced is transparent. The color change occurs due to a structural change in the molecule, in which the double bond between nitrogen and carbon passes to a simple bond. This insaturation changes the entire molecule, resulting in a macroscopic change in the overall color of the biodegraded substance. The DCPIP indicator is applied in a series of electron transfer reactions, including biodegradable substances. Colorimetric methodology applied to oil biodegradation promotes a better handling of different oil microbial biodegrading profiles. Moreover, such rapid and simple colorimetric methodology provides resources on the development of new techniques in effluent treatments, not only during petrol derived oils, but also on other contaminated organic polymeric compounds.

Keywords oil pollution; bioremediation; microbial metabolism; colorimetry; 2,6-dichlorophenol indophenol

1. Remediation of impacted environments

1.1 Oil pollution

Petroleum products are extensively widespread all over the world and their intensive use is strongly connected to the anthropogeneous discharge of hydrocarbons into the environment [1]. Environmental contamination by petrol derivatives has been a subject of study over the past four decades. The leakage of these derivative oils, such as lubricant oils, is capable of harming the environment in many ways [2].

A major concern for petroleum hydrocarbon bioremediation is the presence of heavy compounds such as polycyclic aromatic hydrocarbons (PAHs), asphaltenes and many branched compounds with 20 or more carbon atoms. These heavy hydrocarbon constituents are not easily metabolized by microorganisms and are considered potential health risks due to their possible carcinogenic and mutagenic actions [3].

Furthermore, 1% of all oil consumption is used to produce lubricants [4] and all procedures in lubricant oil production and transport can generate environmental impacts [5]. Hamblin [6] emphasized the percentage of used oil discharged into ecosystems without any treatment as 13% for Europe and 32% for U.S.A. Moreover, lubricant oil can persist for more than six years in some ecosystems, resulting in chronic problems to the biota [7]. Even under laboratory conditions, the complete metabolism of oil by microorganisms takes weeks to months [2].

Lubricant discharge in nature causes continuous concern due to its non-quantified impact and its potential chronic damage [8]. In this context, residual lubricant oil contained in commercial bottles causes significant contamination, because one ton of this automotive oil corresponds to the waste produced from the domestic sewers of 40,000
inhabitants per day. Besides this, only 1.0 L of lubricant is able to deplete the oxygen of 1,000,000 L of water, therefore a city discharge of 200,000 inhabitants is the same as 47 L of residual oil, which affects 47 million L of water [9].

1.2 Remediation processes

Physical and chemical processes are usually used in remediation of contaminated areas. Dispersion, dilution, sorption, volatilization and abiotic transformations are important means of hydrocarbon elimination. However, this type of treatment system requires heavy machinery and the environmental consequences of this pollutant removal may result in massive air pollution [10]. Allowing high efficiency and low costs, bioremediation processes represent an extremely important way of recovering oil contaminated areas among several other cleaning-up techniques [11].

Remediation by physical treatments revealed that, even though the soil is unaffected or unmodified by these treatments, the costs are high, and thus unviable in a large scale.

In spite of this, biodegradation is most often the primary mechanism for contaminant clean-up [12, 13]. Also, for the remediation of hydrocarbon contaminated sites, biological technologies are a widely used, cost efficient and sustainable approach [14].

2. Biological treatment

2.1 Degradation by microorganisms

Bioremediation exploits the ability of some microorganisms to degrade organic contaminants and has been established as an efficient, cost-effective, and environmentally friendly treatment [13; 15]. This process depends on environment conditions and the microbial community structure and, in unfavorable conditions, is generally achieved via bioaugmentation, biostimulation or both [16].

Microbial biotransformation is considered a major environmental technique in treating hydrocarbon pollution in both terrestrial and aquatic ecosystems [17; 18]. This suggests that the biodegradative contribution of the indigenous microorganisms is often significant [13; 19].

Therefore, in order to eliminate or minimize the effects of such contaminations, bioremediation shows up as an effective alternative, which can be used by potentially biodegrading microorganisms [2]. Microorganisms are capable of performing the mineralization of organic chemicals, transforming substances ultimately into CO$_2$, water and biomass [20]. This transformation capacity is of main importance in bioremediation processes, which relies on biodegradation.

The activation of natural degradation potentials in environmental media is the challenge for environmental research that addresses remediation methods. Notwithstanding the widespread application, in situ bioremediation is a site-specific process and feasibility studies are required before full-scale remediation can be applied successfully [21]. Moreover, degradation rates and in particular residual concentrations derived from preliminary studies in the laboratory can be used to predict achievable rates and concentrations in the field [22].

Hence, feasibility studies are a prerequisite for any planned strategy in bioremediation contaminated environments in order to identify limitations towards biodegradation and to predict remediation performance and thereby rule out technologies that may be inappropriate for the clean-up of the site of concern [23; 24]. Additionally, the environmental behavior and chemical-physical properties of lubricant oils are the basis for new fluid developments and for ecological treatment strategies [25].

2.2 Bioremediation of hydrocarbons

Viable methods in biodegradation data generation during biotechnological process application are fundamental in the elaboration of original references about the biodegradability of certain substances [26]. There are many techniques capable of precisely evaluating biodegradation processes, including colorimetric methods. The isolation, characterization and profile of specific bacteria for petrol derived oils biodegradation capacity studies are important when deciding the correct bioremediation strategy.

According to Torstensson [27] and Aichberger et al. [23], preliminary assessments account for three critical prerequisites in bioremediation, namely: (i) the availability of chemicals for the biodegrading microorganisms; (ii) the quantity of these microorganisms; and (iii) their activity level. Other factors are also important in moderating and driving biodegradation, such as temperature, pH, aeration, organic matter and nutrients.

Due to hydrocarbons being widespread pollutants, their hydrophobicity causes a low bioavailability and therefore a particular persistence against bioremediation measures [28; 29]. However, numerous terrestrial and aquatic species of microorganisms possess the ability to degrade these hydrocarbons. In order to increase the bioavailability of these otherwise hardly accessible substrates, microorganisms use strategies like increasing the hydrophobicity of their surfaces or the production of biosurfactants [30].
Long chain hydrocarbons, specifically cyclic alkanes present in petrol derived oils, are difficult for bacteria to degrade [31]. In order to efficiently promote biodegradation, many hydrocarbon degrading bacteria were investigated after isolation in an oil polluted environment [32-35].

Referring to automotive lubricants, biodegradation studies demonstrated that used oil showed considerable assimilation by microbial metabolism when compared to new fluids such as mineral, semi-synthetic and synthetic ones. Automotive lubricant processing in engines modifies its physical-chemical properties due to high pressure and temperature that promote breaks in the hydrocarbon chains. Thus, shorter carbon molecules facilitate microorganism degradation [36; 37].

Lopes et al. [38] studied the toxicity of different lubricant oils (mineral, semi-synthetic and used) before and after biodegradation using *Eruca sativa* seeds (arugula) and *Eisenia fetida* (earthworm) as test-organisms. It was observed that although used oil was the most biodegradable, it was more toxic than new lubricants. Also, as biodegradation proceeded the toxic potential was reduced, but a substantial toxicity remained as hydrocarbons persisted in the soil [39].

Thus, the used lubricant oil hazards are harder to quantify because of its complex composition. Many compounds contribute to high toxicity, but the PAHs formed in service transform used oil into a potential carcinogen. PAHs derive from fuel combustion and lubricant decomposition, therefore their concentration increases in used oils [40].

2.3 Biostimulation

Microbial efficiency in degrading organic compounds is a function of the ability of the microbial degraders to remain active in the natural environment. Hence, increasing the ability of the inoculated microorganisms by bioaugmentation or promoting the activity of indigenous microbial degraders by biostimulation could improve bioremediation [12].

It has been reported that the addition of nutrients has a beneficiary effect on hydrocarbon degradation in the soil by enhancing microbial activity in organic compounds metabolism [39; 41-43]. According to this, a carbon:nitrogen:phosphorous (C:N:P) ratio of 100:10:1 is commonly proposed [44; 45].

2.4 Bioaugmentation

Individual isolates have lower ability than microbial consortia in biodegradation, especially for complex mixtures of compounds such as diesel oil and crude oil [43].

Vecchiolli et al. [46] demonstrated that in addition to the natural presence of hydrocarbon degrading bacteria in soils, exogenous microbial inoculation is able to accelerate the biodegradation whenever conditions are appropriate. They conclude that biodegradation of polluted soils with petroleum hydrocarbons such as accidental spills may be enhanced by indigenous bacteria inoculation. In the same way, Goldstein et al. [47] observed that when species capable of destroying organic compounds are added to the natural environment there is an enhanced biodegradation of these substances. The addition of microorganisms in polluted environments is a technique called bioaugmentation.

Bioaugmentation provides specific microbes in sufficient number to complete the biodegradation process. Over the last few years, contaminating compounds such as insecticides, petroleum compounds and a growing number of toxic organic chemicals have been successfully remediated using bioaugmentation [48-50].

Several advantages are present in bioaugmentation when compared to other techniques: the specific inoculum addition starts the degradation process immediately whereas, in biostimulation, after the nutrients are injected there is a delay in beginning the major biodegradation process [51].

It must be noted that in nature biodegradation capacity is not due to a single microorganism within oils. Metabolic processes in hydrocarbon breaks occur with a succession of species within a microbial consortium. Even non-petrol degrading microorganisms may play an important role in environment oil removal, as the degradation of petroleum leads to a progressive chain of reactions, which leads to intermediate compounds handled by other types of microorganisms [52].

Moreover, different microorganism species have different biodegradation capabilities. Due to this fact, the elaboration of different types of oil biodegradation profiles by different bacteria is an important task for selecting microorganisms in bioremediation processes. In order to accomplish this, screening and evaluation methods adapted to potentially biodegrading bacteria have been established.

3. Redox indicator 2,6-dichlorophenol indophenol

3.1 Redox reactions

Alternative means of determining when reducing conditions exist are provided from redox indicators. Generally, it is observed that oxidized forms are colored and reduced forms are colorless. Due to this fact, color changes can be monitored with a spectrophotometer, evaluating changes in redox indicator absorbance value.

The redox half reaction of a redox indicator is described by Equation 1 below. As the oxidized indicator reacts with a reductant, the absorbance decreases and the reducing power of the sample can be estimated.
\[
\text{Ind}_{\text{ox}} + n\text{e}^- + m\text{H}^+ = \text{Ind}_{\text{red}},
\]

where:
- \(\text{Ind}_{\text{ox}}\) is the oxidized form of the indicator,
- \(\text{Ind}_{\text{red}}\) is the reduced form,
- \(n\text{e}^-\) is the number of electrons transferred, and
- \(m\) is the number of protons transferred - pH dependant [53].

Also, many redox indicators are reversible and couple to a Pt electrode [54]. The redox potential for an indicator \((E_{\text{ind}})\) is determined by the relative concentrations of both oxidized and reduced species and the Nernst Equation 2 below.

\[
E_{\text{ind}} = E^{o}_{\text{ind,pH}} - \frac{RT}{nF} \ln \left( \frac{[\text{Ind}_{\text{red}}]}{[\text{Ind}_{\text{ox}}]} \right),
\]

where:
- \(E^{o}_{\text{ind,pH}}\) is the formal potential of the indicator at the specified pH.

The potential can vary between various indicators and is often a complex function of pH, since many different indicators have groups (i.e. amines) that can be protonated or deprotonated.

### 3.2 Redox within microorganisms

Metabolic pathways within microbial cells are concisely regulated and extremely sensitive to the cell’s need. As a rule, redox reactions and specific redox couples, that cycle between oxidized and reduced states, occur in all of the major metabolic pathways in cells. The concentrations of the redox couples help to regulate the flow of metabolites through these pathways. These intracellular concentrations of redox couples might, likewise, be responsive to receptor agonists, as well as cell nutrients [55]. According to this fact, by using chemical analysis, such concentration changes can be evaluated.

As an example from Rabinowitz et al. [56], some biochemical techniques are used substantially for the direct quantization of NAD+/NADH, NADP+/NADPH, cystine/cysteine, and oxidized/reduced glutathione (γ-glutamylcysteinylglycine, GSSG/GSH), thus, developing probes of cellular redox reactions permit the screening of chemical libraries for compounds affecting redox metabolism. Moreover, for any one redox pair, different potentials may be maintained in different subcellular compartments [57].

### 3.3 Redox indicator DCPIP as an electron acceptor

Hydrocarbon oxidation processes by microorganisms involve redox reactions, in which electrons are transferred to electron acceptors, such as \(O_2\) nitrates and sulfate [58], as seen in Fig. 1. The microbial sulfate-reducing redox is one of the most reducing redox environmental systems. In terms of formal reduction potentials, sulfate reduction to sulfide occurs at about 220mV at pH 7 [59]. A low redox level is achieved in an environment where conditions are completely anaerobic and the terminal electron acceptors (TA) such as NO\(_3\), Mn(IV) and Fe(III) have been fully depleted [60]. Sulfate-reducers use sulfate as the TA and commonly lactate, acetate, or H2 as the electron donor in the process of creating ATP, and producing sulfide [59].

![DCPIP redox scheme, for the catalytic oxidation of sulphide](adapted from Florou [61]).

Common means of measuring sulfate include ion chromatography with a conductivity detector and gravimetric or turbidimetric methods based on Ba(II) as a precipitating agent [62]. Redox environments, including the sulfate-reducing and/or nitrate-reducing conditions in environmental systems, are usually determined by monitoring decreases or increases in sulfate concentration. This way, as seen in Fig. 1, microbial metabolism can also be commonly measured with some colorimetric methods including methylene blue method, iodometric titrimetry or the 2,6-dichlorophenol-indophenol indicator (referred as from this point as DCPIP).

Colorimetric methods are referred to as a low cost and rapid procedure in detecting microbial metabolism occurrence, both in aerobic or anaerobic instances. These methods represent a rapid catalytic microbial based method in which the natural co-substrate (which can be oxygen, sulfates or nitrates) is substituted by a synthetic mediator [63]. Colorimetry is characterized by high levels of microorganisms and mediators, facilitating the fast reaction. Mediators...
basically act as chemical electron carriers that are able to pass through the cell walls of microorganisms, moving between intracellular and extracellular spaces. Once inside the microbial cell, an interaction between mediators and their bioelectrochemical counterparts, such as NAD, NADP, flavoproteins, iron–sulfur proteins, quinones and cytochrome, happens by their interaction with the metabolic process and current redox reactions [64]. Microbial cells catalyze mediator reduction with certain ease when in a very favorable environment. The reduction activity is a sensitive indicator directly coupled to respiration via the electron transport chain, and thus reflects the metabolic status of the cell [65]. DCPIP indicator is included in this category of lipophilic mediator and electron acceptor, capable of probing microbial metabolism.

Thus, by incorporating an electron acceptor such as DCPIP, it is possible to ascertain the ability of a microorganism to utilize a hydrocarbon substrate by simply observing the color change, in this case, from blue (oxidized) to colorless (reduced) according to Fig. 2.

![DCPIP reduction reaction](image)

**Fig. 2** DCPIP reduction reaction.

This technique is aimed at the need to reach a selection of sensitive biocatalysts and mediators capable of detecting the metabolic perturbations caused by various substrates, including toxic ones [66] or biodegrading processes. It is well known that mediators fundamentally work by interacting with the metabolic pathways of the microbial cell. Even though the facts regarding interaction of microbial metabolic pathways with electron transfer mediators is quite an important study, with some important practical usage, few publications are devoted to finding the solution to such an important question as the search for the interaction mechanism between microbial and mediator, and also the origin of the electrons. Thus, more studies are necessary in order to elucidate completely this subject. According to Heiskanen et al. [67], once the interaction mechanism between microbial and mediator is made clear, the mediator will assume the function of an electrochemical probe, as the functionalized mediators would be useful for a better comprehension of the effects of physiochemical reactions occurring within microorganisms in the redox state.

### 3.4 DCPIP characteristics

DCPIP is an enzyme-catalyzed oxidation electron acceptor that is blue in its oxidized form and colorless in its reduced form [68]. Its loss of color is monitored at a wavelength of 600 nm. A peak in the absorbance is observed at 600 nm, as reported by Yoshida et al. [69]. Some studies affirm that DCPIP is unstable in darkness or when exposed to light for a long time [70]. Moreover, the color of the DCPIP indicator in the presence of light is reasonably stable and can be used reliably for some time, especially when adding NAHCO₃ to the dye solution [71].

Additionally, higher concentrations make the color become too dark and the color change induced by reduction with redox reactions is not easily apparent [72]. On the other hand, at lower DCPIP concentrations, the reagent amount is insufficient to signal any perceptible decreases. So, 1g L⁻¹ is the concentration usually selected in many studies [73-75].

A pH value of 7.25 is chosen as satisfactory working stability with DCPIP, since the redox mediator is not sufficiently stable for higher pH values. Moreover, DCPIP’s reactivity is reduced because of its exposure to a very alkaline medium (0.07 M KOH, pH 12). Regarding the temperature effects, the response of the indicator is also established to a maximum response ranging from 297 to 303 K [76]. A calibration curve for DCPIP was obtained from 0.2 to 140 μM with the same spectrophotometer and showed an excellent correlation coefficient (r=0.9997) according to Nakamura et al. [73]. In an earlier report, Yari et al. [71] obtained a similar curve and similar correlation coefficient.

DCPIP is a pH dependant redox indicator, and not only does it change from blue to colorless, it can also turn into a red color at low pH systems, and also in the presence of some other cations e.g. Al³⁺, Fe³⁺, Pb²⁺ and Cu²⁺, which change the pH of the system and act as Lewis’s [53]. Therefore, DCPIP can act as an acid-base indicator, which is reddish at pH< 5.7. Furthermore, at pH >5.7 in alkali and neutral aqueous solutions its color is blue, acting as the electrochemical response of DCPIP [77]. Below a particular potential, it is capable of accepting two electrons from its environment, becoming reduced and giving up its color [71].

### 4. DCPIP Applications

#### 4.1 Discovery and original utilization

The DCPIP redox reaction was originally used and discovered by Vernon and Zaugg in 1960 [78]. They found out that the DCPIP, when ascorbate was present, formed an efficient electron donor couple for photosystem I. Studies involving
individual photoreactions in plant chloroplasts were improved by proper assay systems available and specific photosystems operating in normal photosynthesis. DCPIP brought more details to the knowledge regarding the mechanism of NADP photoreduction by photosystem 1.

Subsequent studies by Yamashita et al. [79] and Nakamoto et al. [80] showed that following the procedure of washing chloroplasts with 0.8 M tris buffer at pH 8.0 prevented any ability of chloroplasts to produce oxygen, however in the presence of DCPIP, along with an excess of ascorbate, photophosphorylation of the non-cyclic type could be restored, which indicated that electrons were entering into the system at the level of photosystem 2. Vernon and Shawl [81] presented some other studies using DCPIP and produced some great achievements in botany regarding oxygen evolution activity within chloroplasts.

4.2 DCPIP related studies

The redox indicator capabilities from DCPIP chemical compound were reported in a series of various studies. In their study regarding the use of *Glucanobacter oxydans* bacteria cells as a biocatalyst at an anode of a biofuel cell with air-based cathode, Alferov et al. [82], used the anode and cathode of the cell made of graphite; DCPIP as an electron transport mediator; and glucose as the substrate to be oxidized. Other uses for DCPIP are: the measurement of mitochondrial complex activities, which determined the activity of succinate–ubiquinone oxidoreductase by the reduction of DCPIP [83]; as the reactions of phytochrome with DCPIP, which is a simple, reliable and easy method for automatable measurement of acetylthiophene during clinical laboratory routines [84]; measurements of the zero current potential of a platinum electrode immersed in solutions of tannins or in wines of various origins with a solution of DCPIP, in order to obtain a global indication for the resistance of some wines to oxidation [85]; the application of an electrochemical sensor, based on a glassy carbon electrode, modified with a cellulose acetate polymeric film bearing DCPIP, for flow injection analysis of sulfide [76]; the kinetic parameters of both human liver and rat purified recombinant PDH E1 proteins were determined utilizing DCPIP [86]; a simple screening protocol using DCPIP to examine the prevalence and the molecular basis of thalassemia in pregnant Laot women [87] and many other original references ranging from botany to medicine [75; 88-90]. A study informed that altered redox homeostasis in control of cancer cell survival and proliferative signaling are also a chemical vulnerability that could be targeted by pro-oxidant redox intervention [91].

5. Biodegradability screening with DCPIP indicator

Pollution by petroleum and its derivates caused by accidental spillage is a concerning problem in the environment. A well known fact is that there is a group of main microorganisms able to consume petroleum hydrocarbons, which are bacteria and fungi [92]. Microorganisms are considered degrading agents of a great variety of substances, corroborating that biological treatment is the most promising alternative for reducing the environmental impact of oil spills [93].

Product composition when released into the environment begins to change immediately because of various biochemical processes. The characteristics of discharged oil pollutants may be changed by physical, chemical and biological mechanisms after large periods in altered environmental conditions. Mixed-function oxidase systems present a great catalytic versatility and are also widely distributed in the environment, taking part in the biotransformation of a wide variety of both exogenous and endogenous organic compounds [94]. Biodegradation involves a list of monooxygenation reactions that are catalyzed, including heteroatom oxygenation and dealkylation, alphatic hydroxylation, desaturation, oxidative group migration and many other modes of inactivation [95]. Thus, the use of DCPIP redox indicator presents a rapid, simple and low cost tool for evaluating capability of different microorganisms to degrade different oils [74; 96].

The biodegradability of petrol derived oils can be verified using the technique based on the DCPIP redox indicator according to what was discussed up to this point. It is important to remember that the principle of this technique is that, during the microbial oxidation of the carbon source, electrons are transferred to electron acceptors such as O₂, nitrates and sulfate. Most petroleum-related hydrocarbons are readily degraded via aerobic microorganisms although a number of studies have shown that in the absence of oxygen alternate electron acceptors such as nitrate, manganese (IV), iron (III), sulfate and carbon dioxide are used [21; 97-101]. By incorporating an electron acceptor such as DCPIP to the culture medium, it is possible to ascertain the ability of the microorganism to utilize the substrate by observing the color change of DCPIP from blue (oxidized) to colorless (reduced) in a screening technology first reported to be applied in oil biodegradation by Hanson et al. [96]. This technique had also been successfully employed in several studies presented below.

Enzyme induction DCPIP activity and the study of the mycelium of *Aspergillus ochraceus* in a kerosene biodegradation were also evaluated. The subject extract was used as an enzyme source for investigation of the induction of NADPH-DCPIP reductase, aminopyrine N-demethylase and kerosene degradation activity, therefore evaluating biodegradation. [102].

A survey of petroleum-degrading bacteria was carried out in India to evaluate distribution of naturally occurring petroleum-degrading aerobic bacteria. Some genera, such as *Pseudomonas, Mycobacterium, Klebsiella, Acinetobacter,*
Micrococcus, and Nocardia were found to be the most common petroleum degraders. Other heterotrophic bacteria included several species of Escherichia, Klebsiella, non-oil-degrading Pseudomonas, Vibrio, Streptococcus, Staphylococcus and Bacillus. Following preliminary selection, five strains, showing best growth in medium with oil fraction as the sole carbon source, were chosen for estimation of the efficiency of crude oil biodegradation using DCPIP as one of the bioindicators [103]. The use of this method proved to be reliable enough in experiments to quantify biodegradation kinetics, taking into account the time taken for decolorization of the blue DCPIP to the colorless form. The strains, which decolorized the DCPIP in the quickest time, were chosen as the best oil degraders [103], but the absorbance values in 600 nm could also be used to precisely collect data regarding the biodegradation process through time.

6. Automotive lubricant oils biodegradation: a case study

6.1 Purpose

Alternative means of determining when reducing conditions exist are provided from redox indicators. Generally, it is observed that oxidized forms are colored and reduced forms are colorless. Due to this fact, such color change can be monitored with a spectrophotometer, evaluating changes in redox indicator absorbance value.

The redox half reaction of a redox indicator is described by Equation 1 below. As the oxidized indicator reacts with a reductant, the absorbance decreases and this way the reducing power of the sample can be estimated.

6.2 Material and methods

The biodegradability of automotive lubricant oil (synthetic, semi-synthetic, mineral and used) was evaluated using the technique based on the redox indicator DCPIP. Hanson et al. [96] technique has been employed in other studies [74; 103; 104]. However, it has never been used in automotive lubricant oils and vegetable oils biodegradation evaluation before.

Colorimetric assays were made using Bacillus subtilis CCT 2576 in NA medium cultures conserved at 10°C. The cells were transferred from storage culture tubes and streaked onto a Petri dish with PCA medium. Then, microbial culture reactivated after 48 h were inoculated to 50 mL of BH medium at 35°C for biomass growth. No agitation or darkness conditions were applied. The BH medium contents are, g L⁻¹: MgSO₄, 0.2; CaCl₂, 0.02; KH₂PO₄, 1.0; K₂HPO₄, 1.0; NH₄NO₃, 1.0; FeCl₃, 0.05; according to Difco [105].

After 48 h in BH medium, B. subtilis culture was inoculated to tubes along with DCPIP indicator and the oil under analysis. Tube contents followed Table 1 and the experiment was conducted in triplicate.

Table 1 Colorimetric assays content.

<table>
<thead>
<tr>
<th></th>
<th>DCPIP</th>
<th>BH</th>
<th>B. subtilis inoculum</th>
<th>Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 0</td>
<td>400 µL</td>
<td>7.5 mL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control 1</td>
<td>400 µL</td>
<td>7.5 mL</td>
<td>100 µL</td>
<td>-</td>
</tr>
<tr>
<td>Water 1</td>
<td>400 µL</td>
<td>7.5 mL</td>
<td>-</td>
<td>50 µL of Synthetic Oil</td>
</tr>
<tr>
<td>Water 2</td>
<td>400 µL</td>
<td>7.5 mL</td>
<td>-</td>
<td>50 µL of Semi-Synthetic Oil</td>
</tr>
<tr>
<td>Water 3</td>
<td>400 µL</td>
<td>7.5 mL</td>
<td>-</td>
<td>50 µL of Mineral Oil</td>
</tr>
<tr>
<td>Water 4</td>
<td>400 µL</td>
<td>7.5 mL</td>
<td>-</td>
<td>50 µL of Used Oil</td>
</tr>
<tr>
<td>Inoculum 1</td>
<td>400 µL</td>
<td>7.5 mL</td>
<td>100 µL</td>
<td>50 µL of Synthetic Oil</td>
</tr>
<tr>
<td>Inoculum 2</td>
<td>400 µL</td>
<td>7.5 mL</td>
<td>100 µL</td>
<td>50 µL of Semi-Synthetic Oil</td>
</tr>
<tr>
<td>Inoculum 3</td>
<td>400 µL</td>
<td>7.5 mL</td>
<td>100 µL</td>
<td>50 µL of Mineral Oil</td>
</tr>
<tr>
<td>Inoculum 4</td>
<td>400 µL</td>
<td>7.5 mL</td>
<td>100 µL</td>
<td>50 µL of Used Oil</td>
</tr>
</tbody>
</table>

Control assays (C0-1) evaluated interactions of the assay components and DCPIP indicator. C0 contained only DCPIP indicator and BH medium. On the other hand, C1 presented DCPIP indicator, BH medium and B. subtilis inoculum; and was done to determine inoculum influences in DCPIP through time. Another series of blank assays, named “Water” contained DCPIP indicator, BH medium and different types of oils (W1-4). Finally, “Inoculum” assays were made up of DCPIP indicator, BH medium, B. subtilis inoculum and the different oils (I1-4).

The absorbance of all the assays was measured by Hach® DR 2500 spectrophotometer. Data was collected three times per day, totaling 230 h. Then, absorbance values were compared between different assays.

6.3 Results

The colorimetric analysis demonstrated that the biodegradation was successful. It occurred in all the oil containing tubes, due to the general pattern whereby DCPIP loses of color.

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The control assays (C0 and C1) kept the blue color, whereas the oil containing assays passed through the biodegradation process (W1-4 and I1-4). However, it was noticeable that the biodegradation time necessary for the blue color to completely vanish was different between the different types of oils.

In the oil containing assays without \textit{B. subtilis} inoculum, total biodegradation times for synthetic (W1), semi-synthetic (W2), mineral (W3) and used (W4) were 210 h, 162 h, 138 h and 89 h, respectively. For the “Inoculum” assays, the DCPIP color changes were 138 h, 125 h, 75 h and 87 h for synthetic (I1), semi-synthetic (I2), mineral (I3) and used oils (I4). For example, Fig. 3 is representing the DCPIP decolorization process during the incubation time for control and synthetic oil assays.

![Fig. 3](image)

According to results and Fig.3, \textit{B. subtilis} inoculum assays demonstrated a faster biodegradation compared to assays without the bacteria. Even though no biomass was inoculated, biodegradation occurred in “Water” assays probably due to previous microorganism presence in oils. Moreover, the total biodegradation time observed generally followed an ascendant order: synthetic oil, semi-synthetic oil, mineral oil and used oil.

Regarding quantitative data, the absorbance readings of the DCPIP indicator are represented in Fig. 4.

In relation to Fig. 4a, a small decrease in DCPIP concentration can be noticed even in control assays. This decrease indicates an overall pattern of natural disintegration of DCPIP chemical structure through time. Therefore, it cannot be due to the \textit{Bacillus subtilis} inoculum, since the DCPIP concentration decreases equally in both C0 and C1. Considering this, even though the DCPIP concentration tends to naturally decrease, the tubes containing oils reduce the DCPIP concentration even further because of biodegradation.

The results of Water and Inoculum assays for the different oils are presented in Figs. 4b, 4c, 4d and 4e.

![Fig. 4](image)

**Fig. 3** DCPIP decolorization process through time (3, 137 and 210 hours, respectively) as observed in synthetic lubricant oil biodegradation. From left to right, the five test tubes are: distilled water; C0; C1; Water1 and Inoculum1.

**Fig. 4** DCPIP concentration quantified at 600 nm absorbance (a) in C0 and C1 assays - without oils; and in oil containing assays without (Water) and with (Inoculum) \textit{Bacillus subtilis} (b) synthetic - 1; (c) semi-synthetic -2; (d) mineral - 3 and (e) used - 4.
At the outset, the complete loss of color happened in the last measurement point plotted. So, the DCPIP was considered reduced as a result of the biodegradation when the absorbance value led to a DCPIP concentration below 0.030 g L\(^{-1}\). Values below this concentration could not be measured by this spectrophotometric method.

Another consideration regarding oils containing assays (Figs. 4b, 4c, 4d and 4e) is that all the decreasing curves approach a linear model, except Fig. 4b and Fig. 4c, which are better fit to an exponential decay model.

As a pattern observed in the assays, DCPIP quantification demonstrated a tendency through time (Fig. 4a) but it was not as significant as the DCPIP concentration reduction attributable to oil biodegradation by B. subtilis. These assays presented a DCPIP absorbance decrease, which indicates a positive interaction of the bacteria in oil degradation, since all “Inoculum” assays yielded a faster biodegradation. Also, “Water” assays presented a DCPIP concentration reduction as they already previously contained microorganisms in oils.

6.4 Discussion

As a general pattern, used oils tend to be more easily biodegraded, biodegrading faster than the non-used retail available samples. On the other hand, the synthetic and semi-synthetic automotive lubricant oils took the longest time (125 and 138 h, respectively) to completely change the color of DCPIP from blue to transparent, even with B. subtilis inoculum. The mineral oil had an intermediate biodegradation value between the automotive lubricant oils.

Concerning the biodegradation of automotive lubricant oils, the process had been previously reported using different methodologies, such as respirometric studies. In Lopes and Bidoia [36], the synthetic lubricant oil presented a better biodegradation performance than the mineral and semi-synthetic lubricant oils.

Eisentraeger et al. [25] observed that used oils were more biodegraded than new ones, and proposed that this is due to changes in the chemical structure of the hydrocarbons, facilitating the interactions between the oil and the microorganisms. Also, the use of lubricant oil in engines, with high pressure and temperature, break the long hydrocarbon chains, thus supporting microbial activity.

In conclusion, this rapid and simple colorimetric methodology applied to oils biodegradation promotes some discussion leading to a better handling of contaminated water medium by oils and their respective biodegrading profile. Thus, it promotes the development of new techniques in water treatment. From this data, the automotive lubricant oils biodegradation occurs because hydrocarbons and organic polymers are used as metabolic substrates by microorganisms.

Also, some lubricant oil compounds such as cyclic alkanes are not biodegraded by only one microorganism. Hence, a microorganism consortium allied to a known potentially oil metabolizing microorganism such as B. subtilis will also perform an important role in degrading these compounds [106]. Therefore, a Bacillus subtilis inoculum yields a better oil biodegradation.

7. Final considerations

Microorganisms represent an extremely reliable way to biodegrade petrol derived substances. This ability is highly exploitable during bioremediation processes to ultimately free the environment from subsequent pollutants derived from these hydrocarbons. The development of efficient techniques in order to obtain biodegradation data is a fundamental tool when proposing different strategies for bioremediation of polluted areas. DCPIP redox indicator colorimetric technique proved itself to be a rapid, easy and low cost method when detecting microbial metabolism from carbon and other nutrients sources, including hydrocarbons. To ascertain microbial ability to utilize hydrocarbon substrates by simply observing the color change of DCPIP, in which the quickest decolorization time represents the best oil degradation, is a major breakthrough in biodegradation studies. Further studies in the applicability of this method and other different redox indicators are recommended in order to reach a wide and precise selection of mediators capable of detecting the metabolic perturbations caused by many types of substrates, even though DCPIP usage has already been successfully reported for a wide range of situations.

Acknowledgements The authors thank FUNDUNESP; FAPESP; CAPES and CNPq for support.

References


