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Potential of Fungi Isolated from Diesel Contaminated Soil to Degrade Diesel

Oluwafemi A. Oyewole[1*], Ramat O. Raji[1], Suzan K. Okeke [2], Innocent O. Musa[1], Gift N. Chukwuma[1] and Japhet G. Yakubu[1]

[1]*Department of Microbiology, Federal University of Technology Minna, Nigeria*
[2]*Department of Biological Sciences, The Federal Polytechnic Bida, Nigeria*
**Corresponding author, e-mail address: oa.oyewole@futminna.edu.ng*
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Abstract

Diesel is a complex mixture and a common pollutant, consisting mainly of aliphatic hydrocarbons ranging from C_9 to C_{23} and a number of aromatic compounds. The main purpose of this study was to isolate fungi from diesel contaminated soil for biodegradation of diesel contaminated soil. Two fungal species (*Aspergillus flavus* and *Saccharomyces cerevisiae*) were isolated from a diesel contaminated site using cultural and biochemical characterisation. The isolates were screened for their ability to utilize diesel as carbon source. The biodegradation rate of the fungi isolated was measured by determining the optical density using spectrophotometry. The fungal isolates were further tested for utilisation of hydrocarbons using mineral salt medium (MSM) supplemented with diesel in varying concentrations (1%, 2%, 5% and 10%). The optimum pH for biodegradation (4, 6 and 8) was determined. Both isolates degraded best at 5% diesel concentration with optical densities of 1.751 and 1.546 for *A. flavus* and *S. cerevisiae*, respectively. The optimum pH for *A. flavus* was pH 8 (optical density 1.390), while the optimum pH for *S. cerevisiae* was pH 6 (optical density 1.234). The results of this study showed that the isolates were able to degrade 5% diesel concentration at optimum pH of 8, and can be developed to remediate diesel contaminated environment.

Keywords: Diesel; biodegradation; *Aspergillus flavus; Saccharomyces cerevisiae*.

Introduction

Diesel is a complex mixture and a common pollutant (Osinowo et al. 2020). It consists mainly of aliphatic hydrocarbons ranging from C_9 to C_{23} and a number of aromatic compounds. The environmental impacts of diesel when spilled include reduction of plant growth through direct toxic effects on the plants, reduced germination, and unsatisfactory soil conditions due to insufficient aeration of the pore space between the soil particles (Osinowo et al. 2020). Thus, the biological functioning of microbial communities, which are central to the biogeochemical cycling of ecosystem, is truncated. This consequently affects the productivity of such ecosystems (Nwinyi et al. 2014, Osinowo et al. 2020).

One of the most common contaminants in the environment is crude oil and its derivatives and due to their wide spread occurrence, they pose severe risks to human health and water bodies (surface as well as ground), and so, intense remediation practices are required at the contaminated sites (Oyewole et al. 2020). Globally, there are rising concerns about the risks of environmental contamination from the exploration, transport and storage of petroleum. In Nigeria, crude oil production capacity increased to about 2.8 million barrels

per day. Nigeria ranks as one of the Africa's largest producers of oil and the sixth largest oil producing country in the world (Nwinyi et al. 2014). In Nigeria, the hub of oil exploration and transportation is the Niger Delta. This ecological zone and its surrounding offshore areas supply above 80% of the country's crude oil. In addition, the petroleum and petrochemical products are produced, refined and handled in this region. With the over-dependence on the vehicles for the transportation of the petroleum products, accidental spills are unabated. Fuel stations and other storage containers have life spans that are minimal, and as a result, soil and aquifer contamination cases originate from these sources (Raju and Scalvenzi 2017, Osinowo et al. 2020, Oyewole et al. 2020).

In large concentrations, the hydrocarbon molecules that make up crude oil and petroleum products are highly toxic to many organisms, including human beings. It is estimated that between 1.7 and 8.8 million metric tons of oil are released into the world's water and soil every year, 90% of which are directly related to human activities including deliberate waste disposal. It is also estimated that about 30% of the spilled oil enters freshwater systems (Dadrasnia and Agamuthu 2013).

Numerous anthropogenic sources can be distinguished such as oil extraction and treatment fields; transportation fuels accidents, leakage from underground storage tanks, petrochemical industry activities and release from oil refinery sites (Oyewole et al. 2020). To reduce this contamination, different chemical, physical and biological treatment methods are considered. The choice of remediation methods depends on several parameters such as the type of pollutant and its characteristics (the physicochemical nature of the pollutant and its toxicity), the properties of contaminated site (the pollution source, and the nature of the site) and the type of the pollution (old or recent). The treatment of a polluted site is carried out only after evaluating the type of pollutant, the environmental and human-associated risks and the treatment feasibility and predicted efficiency (Mahjoubi et al. 2018, Oyewole et al. 2019, Osinowo et al. 2020).

Depending on its location, *in situ* and *ex situ* contaminated site remediation methods are distinguished. However, bioremediation is considered as one of the best environment-friendly way to remove hydrocarbons presenting several advantages compared to other methods. It is a natural, efficient and economic method (Oyewole et al. 2020). In addition, it converts hydrocarbons into less toxic compounds through metabolic and enzymatic reactions. The biodegradation is mainly carried out by bacteria, yeast and fungi. Bacteria represent the major class of microorganisms involved in the degradation of hydrocarbons (Mahjoubi et al. 2018, Oyewole et al. 2020).

In the last decade, the roles of fungi in bioremediation of hydrocarbons have been increasingly recognized. Various authors have highlighted the ability of fungi, mainly saprotrophic and biotrophic basidiomycetes, to degrade or to transform toxic compounds. Mycoremediation is the bioremediation technique, which employs fungi in the removal of toxic compounds that utilize enzymes for the degradation of a large variety of pollutants (Bosco and Mollea 2019). Fungi are well known for their ability to colonize a wide range of heterogeneous environments and for their ability to adapt to the complex soil matrices, even at extreme environmental conditions.

Fungal hydrocarbon degradation is mostly an extracellular process, involving the release into the environment of active broad-specificity oxidoreductase enzymes, such as laccases, manganese peroxidases and lignin peroxidases (Correa-García et al. 2018). In nature, these enzymes are mainly used to degrade lignin (a cross-linked phenolic polymer), but their low specificity also allows them to degrade other phenolic compounds, such as the ones found in petroleum hydrocarbons.

Petroleum pollution has become a serious environmental problem worldwide, which can cause harmful damage to the environment and human health. This pollutant is introduced into the environment from both natural and anthropogenic sources. Diesel is heavy oil used as fuel for internal combustion in diesel engines, as a burner in fuel heating installations, such as furnaces and for enriching water gas to increase its luminosity. Virtually all industries use clean water sources from boreholes, rivers, oceans and or sea for industrial productivity. If much of these water bodies are polluted by oil spills, it jeopardizes industrial plants such as power, desalination, nuclear and water treatment plants. This is because, treating heavy oil polluted water is expensive and complex (Saleh et al. 2017).

Highly concentrated oil spillage is so complicated during cleaning processes. Similarly, cleaning oil spills requires very huge capitals to achieve certain cleaning effectiveness (Akpomuvie 2011). Thus, the outcome of the cleaning process may not attain 100% efficiency. Some of the effects of oil spillage include the loss of the crude oil itself, and the money that could have been realized economically from the spilled oil (Saleh et al. 2017). Tourism sector is seriously hampered, as most of the game reserves, ranches, mangroves/swamp areas, and water springs, as well as water related recreational activities virtually or completely die off. Similarly, health and safety of the environment are jeopardized (Saleh et al. 2017). The increasing rates of diesel-contaminated soils have made this research a necessity to exploit other means of eliminating this contaminant from the soil, which maintains the environment in its natural state, other than the use of other conventional methods. These conventional methods often lead to the transformation of this contaminant from one toxic form to another (Saleh et al. 2017). The aim of this research was to evaluate the bioremediation ability of diesel by fungi isolated from diesel contaminated soil.

Materials and Methods

Collection of soil samples

Soil samples were collected from a location mounted with a diesel powered generator in Bosso, Minna, Nigeria in a sterile soil auger and taken to the Microbiology Laboratory, Department of Microbiology, Federal University of Technology Minna, Nigeria.

Isolation of fungi

Soil samples were mixed to homogeneity and sieved to remove stones, and soil debris. Sieved soil samples were serially diluted up to 10^{-5} dilution. Potato Dextrose Agar (PDA) was prepared and autoclaved at 121 °C for 15 min, which was later supplemented with 50 mg/ml ampicillin and 10% of filtered sterile used engine oil. One (1) ml of dilution from 10^{-3}, 10^{-4} and 10^{-5} were poured on Potato Dextrose Agar (PDA) plates and left for incubation at 28 °C for a period of three days (Thenmozhi et al. 2013, Tiwari and Saraf 2017). Different colonies were observed in the plates after a period of incubation, each fungal colony was sub cultured on a fresh PDA medium both in plates and slants for identification.

Screening of diesel degrading fungi

A selective medium (oil agar medium) also known as mineral salt medium (MSM) was used for screening diesel degrading fungi. The fungal isolates were transferred to a Potato Dextrose Broth (PDB) and incubated for 24 h. The composition of the MSM was potassium dihydrogen phosphate (0.16 g), magnesium sulphate (0.08 g), sodium chloride (0.04 g), calcium chloride (0.01 g), ammonium nitrate (0.2 g) (Sukumar and Nirmala 2016). The MSM was deposited into two test tubes, inoculated with 1ml suspension of each isolate, supplemented with 1% diesel and incubated. A control was set up without fungal inoculum. The growth was monitored for 21 days using spectrophotometry. Fungal isolates that gave heavy sporulation, more abundant aerial mycelium and greater colony diameter were considered as organisms utilizing hydrocarbons, which were later confirmed by

further screening as described by Thenmozhi et al. (2013) and Tiwari and Saraf (2017).

Identification of diesel degrading fungi

The indigenous fungi exhibiting degradation of petroleum were grown on PDA. The observed characteristics of the isolates were recorded. Morphological identification was performed for the selected fungal strains. Both macroscopic (cultural characteristics) and microscopic (lactophenol cotton blue staining) examinations were performed on the isolates. The results were confirmed by comparing their morphology and cultural characteristics with descriptions given by Thenmozhi et al. (2013) and Tiwari and Saraf (2017). Various sugar fermentation tests were carried out that included glucose, maltose, sucrose, lactose, xylose, raffinose and galactose.

Determination of pH conditions for fungal biodegradation of diesel samples

Potato dextrose broth was prepared and dispensed into different test tubes and the fungi colonies isolated were inoculated into the test tubes and incubated for 24 h. Mineral salt medium was also prepared and conditioned to pH 4, 6 and 8 so as to determine the best pH for biodegradation of diesel. The 0.1 ml of diesel oil was added as the sole carbon source and 1 ml of suspension from 24 h broth was added into pH 4, 6 and 8 test tubes. The medium was conditioned to pH 4, 6 and 8 because pH 4 is acidic, pH 6 is almost neutral and pH 8 is basic so as to determine the optimum pH at which each fungus can biodegrade diesel oil. To achieve this pH, hydrochloric acid was added to reduce the pH level of the medium and sodium hydroxide was added to increase the pH level to basic level. The optical density of the residual diesel oil was scanned using UV-Vis spectrophotometer using a wavelength of 545 nm at an interval of 3 days. The extent of degradation was recorded by observing the change in the absorption of the diesel oil whether there was increase or decrease in absorbance in the UV-vis spectrophotometer when compared to the control (Kumar and Manjunatha 2015).

Effect of hydrocarbon concentration on degradation rates

The biodegradation potential was determined by preparing MSM and potato dextrose broth. Twenty (20) ml of PDB were dispensed into two test tubes and the two fungal isolates were inoculated in the test tubes containing PDB and incubated for 24h. After incubation, 1 ml of the suspension of each fungal isolate was dispensed into the test tubes containing the MSM. The percentages of diesel used were 1%, 2%, 5% and 10%. The optical density of the residual diesel oil was determined using UV-Vis spectrophotometer (Model 752, China) at a wavelength of 545 nm. The extent of degradation was recorded by observing the changes in the absorbance readings (optical density) on UV-Vis spectrophotometry of the diesel oil whether there was increase or decrease in the optical density when compared to the control (Sukumar and Nirmala 2016).

Results

Fungi isolated from diesel contaminated soil

Two fungi (*Aspergillus flavus* and *Saccharomyces cerevisiae*) were isolated from the diesel contaminated soil. The first fungi colony appeared yellowish green in color and the growth form had powdery masses of yellow green spores on the upper surface. Microscopy showed septate hyphae with long conidiophores, which have rough velvety textures. Macroscopy showed very rapid rate of growth maturing at 3 days. The name of the fungi was confirmed to be *Aspergillus flavus* (Plate 1). The second fungi colony appeared dull cream in color. They were flat, smooth, moist, glistening and dull cream in color. The results of the various sugar fermentation tests are recorded in Table 1. The identity of the fungi was confirmed to be *Saccharomyces cerevisiae* (Plate 2).

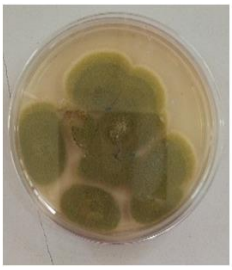

Plate 1. *Aspergillus flavus*

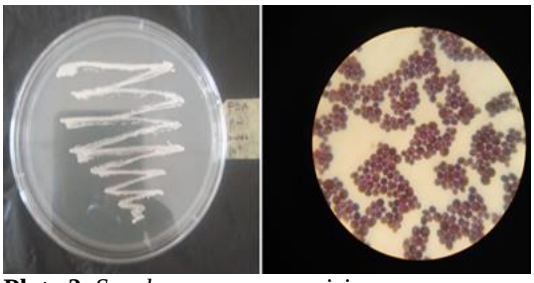

Plate 2. *Saccharomyces cerevisiae*
(a) Macroscopic and (b) microscopic

Table 1: Sugar fermentation ability of *S. cerevisiae*
Keys: + = positive, – = negative, Gluc = glucose, Lact = lactose, Fruc = Fructose, Suc, Sucrose,

Isolates	Gluc	Lact	Fruc	Suc	Malt	Sobit	Manit	Xyl	Rham	Raffin	Galac
S. cerevisiae	+	–	+	+	+	–	–	–	–	+	+

Malt= Maltose, Sobit = Sobitol, Manit = Mannitol, Xyl = xylose, rham = rhanose, raffin = raffinose, galac = galactose.

Screened isolates with diesel degradation potential

The isolates (*Aspergillus flavus* and *Saccharomyces cerevisiae*) grew on the MSM. The growth of the fungal isolates in the medium is shown in Figures 1 and 2. The growth of the *A. flavus* (Figure 1) was higher compared to that of the control. The highest growth was observed after 15 days of incubation. However, in the case of *S. cerevisiae*, the highest growth was recorded on the 12th day (Figure 2).

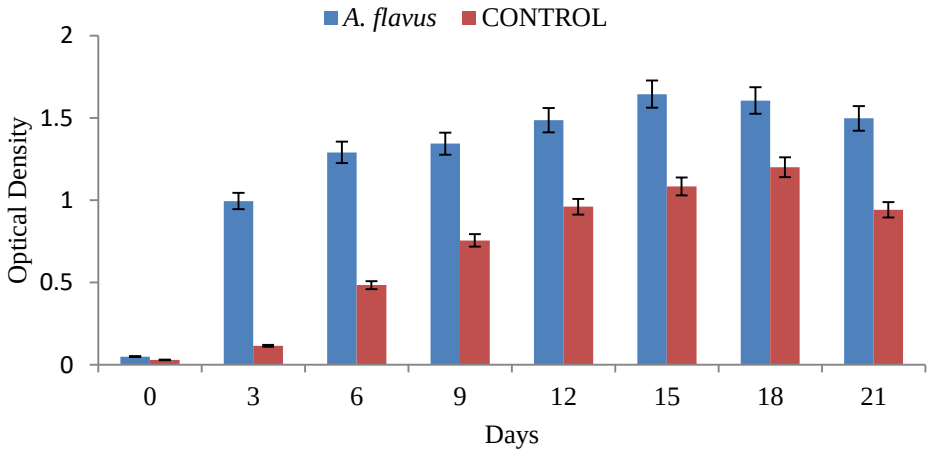

Figure 1: Growth of *A. flavus* on MSM.

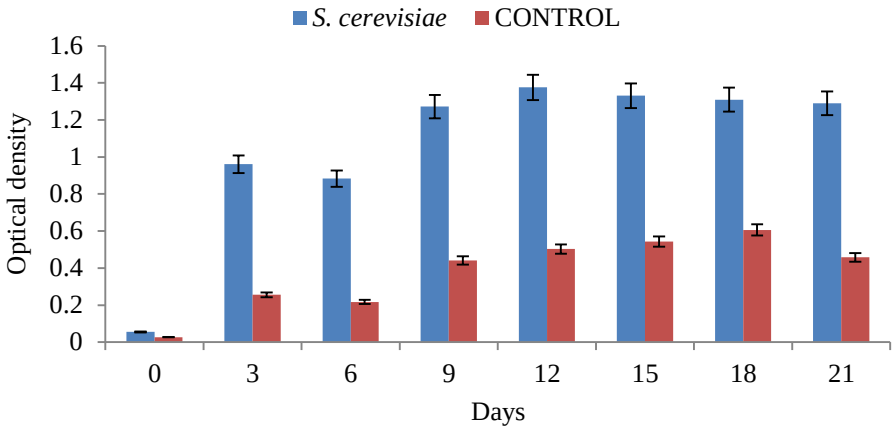

Figure 2: Growth of *S. cerevisiae* on MSM.

pH conditions for fungal biodegradation of diesel samples

The effects of pH (4, 6 and 8) used for bioremediation using *A. flavus* and *S. cerevisiae* are represented in Figures 3 and 4. The *A. flavus* utilized diesel best at pH 8 on the 15th day with optical density of 1.390.

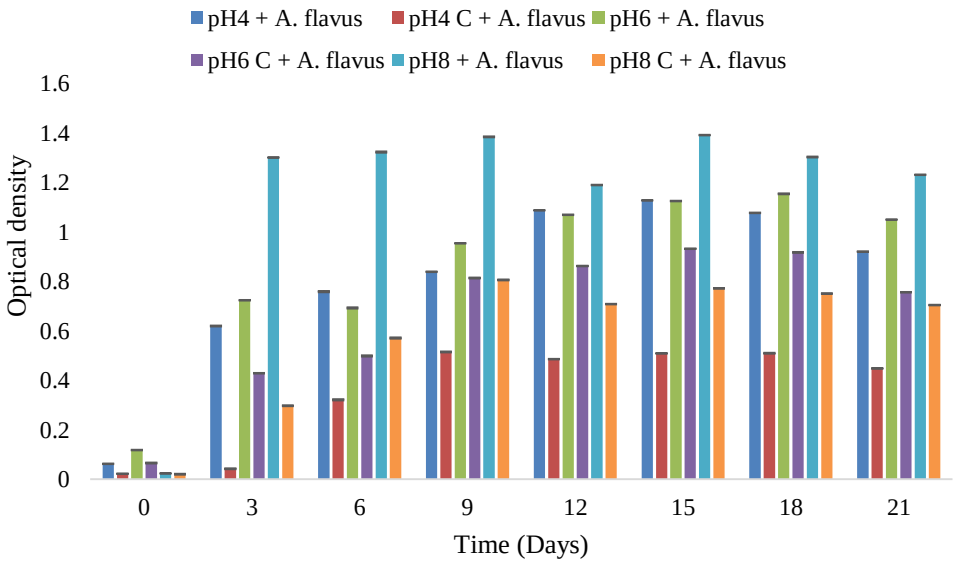

Figure 3: Optical readings of MSM inoculated with *A. flavus* at different pH.

Figure 4 is a chart showing the optical density for diesel utilization by *S. cerevisiae* at different pH values. *S. cerevisiae* utilised diesel best at pH 6 on the 15th day with optical density of 1.243.

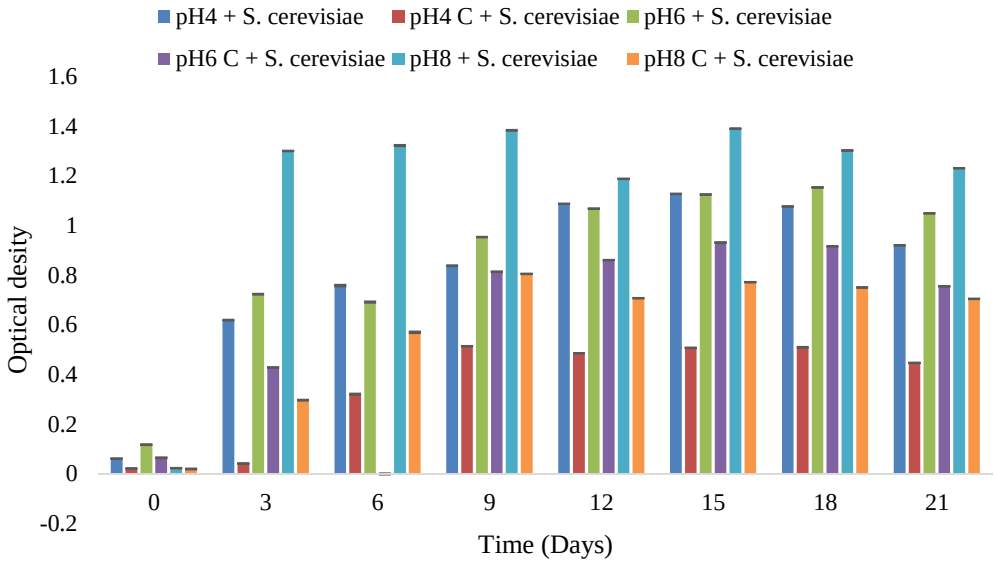

Figure 4: Optical readings of MSM inoculated with *S. cerevisiae* at different pH.

Effect of diesel concentration on bioremediation of fungi

A. flavus utilised diesel best at 5% on the 21st day and *S. cerevisiae* utilised diesel best at 5% on the 18th day (Figure 5).

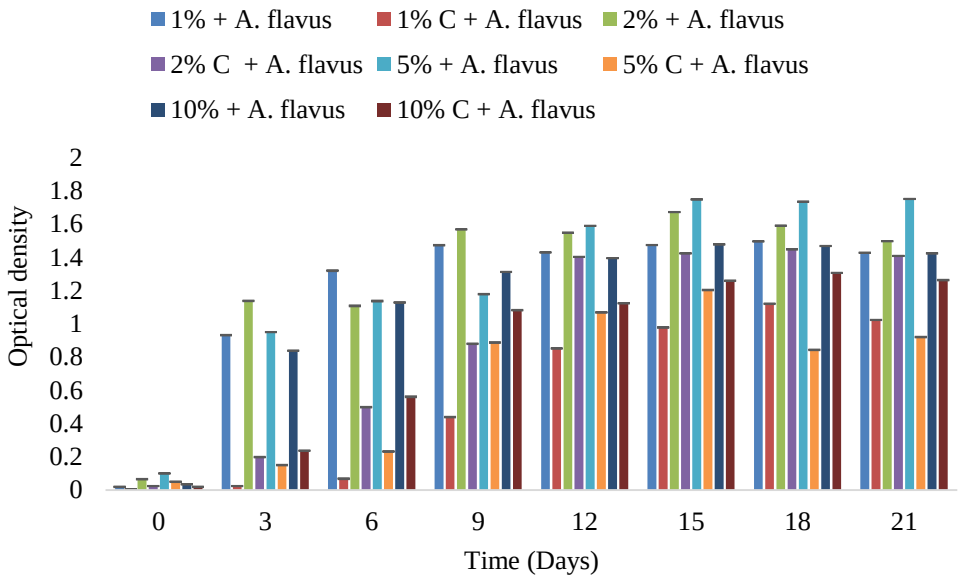

Figure 5: Hydrocarbon concentrations on degradation rates by *A. flavus.*

Figure 6 is a chart showing the diesel utilization readings at 1%, 2%, 5% and 10% for *S. cerevisiae* in which *S. cerevisiae* utilised diesel best at 5% on the 18th day.

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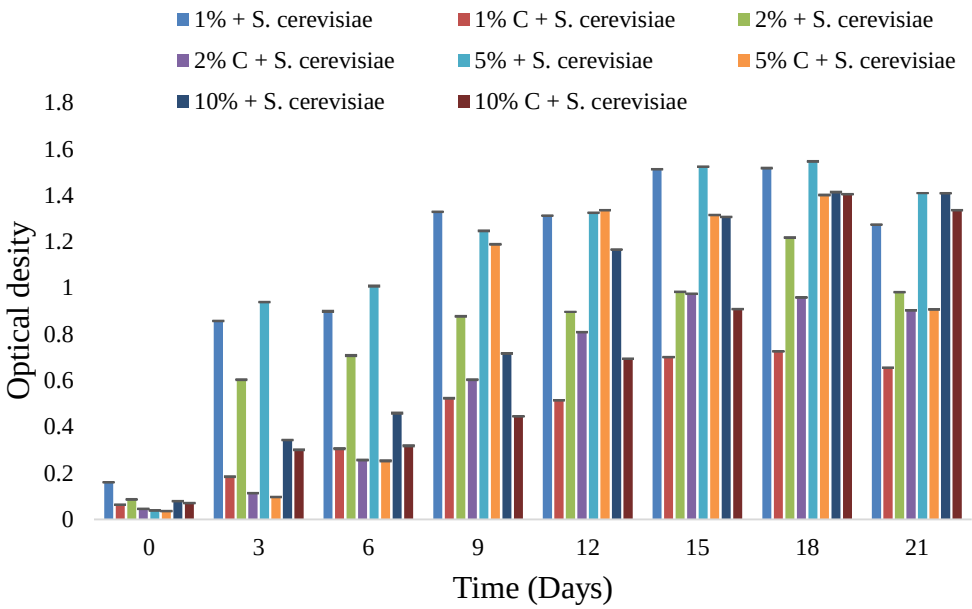

Figure 6: Hydrocarbon concentrations on degradation rates by *S. cerevisiae*.

Discussion

The results obtained in this research showed that *A. flavus* and *S. cerevisiae* isolated from the diesel contaminated site were capable of degrading the diesel. This could be due to the presence of diesel degrading enzymes such as laccases, manganese peroxidases and lignin peroxidases (Correa-García et al. 2018). This report is similar to previous findings by other researchers (Burghal et al. 2016, Al-Jawhari 2016, Ekhaise and Nkwelle 2011).

The *A. flavus* had the highest optical density on the 15th day, while *S. cerevisiae* had highest optical density on the 12th day. This is also similar to the study carried out by Tiwari and Saraf (2017); in their study, *A. flavus* also showed highest optical density.

A. flavus had the highest degradation rate at 5% concentration on day 21 with optical density of 1.751, while *S. cerevisiae* had the highest rate of degradation at 5% concentration on day 18 with optical density of 1.546. Ahirwar and Dehariya (2013) also carried out a similar study using the same method but at a wavelength of 600 nm for optical density, according to which *A. flavus* also gave the higher percentage of degradation. This could be due to the presence of spores and mycelium when compared to *S. cerevisiae* (a yeast). The result in the present study in which *A. flavus* was the more potent diesel degrader agrees with the result of Dutta and Singh (2016) who reported *A. flavus* among those fungal species capable of degrading oil. Tiwari and Saraf (2017) also reported some species, which possessed higher degradation potentials of petroleum products among which was *Aspergillus* species.

There was growth at pH 4, 6 and 8 by *A. flavus* and *S. cerevisiae.* However, the best growth for *A. flavus* was at pH 8 on day 15 with optical density of 1.390, while poor growth was at pH 4 on day 15. The best growth for *S. cerevisiae* was at pH 6 on day 15 with

absorbance reading of 1.234 and poor growth was at pH 4 on day 18. This result is relatively similar to that of Kiama et al. (2015), in which there was optimal growth at pH 5,7 and 9 and the best pH from the study was at pH 9 while poor growth was at pH 5.

An observation from this study shows that there was an increase in the growth of the isolates from day zero (0) to day 18, but on the 21st day, the growth decreased. This may be due to reduction of carbon that is used for their energy and growth. An interesting observation generated in this study was that the fungi isolated had increased growth rates in the media containing diesel oil. This result may be due to the fact that the fungi isolates were able to use the hydrocarbons as substrates for growth by likely releasing extracellular enzymes and acids, which are capable of breaking down the long chains of carbon and hydrogen thereby converting petroleum to simpler forms that can easily be absorbed by the fungi for their nutrition and growth.

Conclusion

Aspergillus flavus and *Saccharomyces cerevisiae* were obtained from diesel contaminated soil and showed capacity to degrade diesel with a higher capacity by *A. flavus*. These isolates possessed the potentials to be utilized as possible diesel remediation agents.

Conflict of Interest

No conflict of interest was declared by the authors.

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