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Research Article

pH-dependent Heavy Metal Toxicity Differentials in Fungal Isolates during Biodegradation of Spent Engine Oil

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Abstract: Indiscriminate disposal of spent engine oil is a global concern because of the numerous health risks to animals and humans following exposure. Indigenous fungi were isolated from soil samples polluted with spent engine oil (SEO) at Mgbuka-Nkpor, Nigeria. They were identified based on their cultural and microscopic characteristics and confirmed using their 18Sr RNA gene sequence. A phylogenetic tree of the isolates was constructed using the neighbour joining method. Heavy metal analysis of spent and new engine oil was conducted using Varian AA240 Atomic Absorption Spectrophotometer. The effect of varying concentrations of heavy metals on SEO degradation by the isolates at different pH levels was also determined. Two fungi isolates were obtained from this study. They were identified and confirmed as *Candida tropicalis* and *Aspergillus clavatus*. The concentration of the heavy metals (zinc, lead, cadmium and copper) was significantly higher ($P < 0.05$) in spent engine oil when compared to the new engine oil. There was negative inhibition (stimulation) in the media containing both the single and mixed culture of the isolates, in the presence of cadmium at pH 5.5. Moreover, highest stimulation (20%) in SEO degradation was recorded in the presence of 10 mg/l copper at pH 5.5, in the media containing *A. clavatus*. These results showed that the pure and mixed culture of the isolates (*C. tropicalis* and *A. clavatus*) have promising potential for effective bioremediation of spent engine oil polluted soil co-contaminated with cadmium and copper at pH 5.5.

Keywords: Biodegradation; spent engine oil; pH-dependent; heavy metal; toxicity

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Introduction

Environmental pollution is assuming an alarming rate worldwide. This problem might not be unconnected with the rapid industrialization, population growth and technological advancement being experienced in most parts of the world today. A wide range of potential hazardous substances is released on a daily basis which contributes to the global pollution burden. For example, heavy metals result from industrial wastes; electronic wastes etc, and pose serious threat to both man and animals if not properly abated [1].

Spent oil is oil that has been used and obtained after servicing and draining from industrial machines and automobiles [2]. It is noteworthy, that spent oil is similar to unused oil, except that additional chemicals and metals such as lead, manganese, iron, tin and silicon have been added to the spent oil, due to high temperature and pressure of the operating engines where the oil serves as engine lubricant [3]. In addition, these added chemicals impurities contribute significantly to chronic hazards because of their solubility in soil surface and groundwater [3, 4].

Studies on the effects of metals on organic pollutant biodegradation are not extensive, but the available few demonstrate that metals have the potential to inhibit pollutant biodegradation [5]. These metals however, can inhibit various cellular processes and their effects are often concentration-dependent and also vary in their individual toxicity [6]. The impacts of metals (Cadmium, Nickel, Zinc, Mercury and Chromium) on litter decomposition, methanogenesis, acidogenesis and biomass generation have all been studied [5, 7]. Olaniran *et al*. [8] reported the impact of zinc, lead, copper and manganese on crude oil biodegradation by a *Micrococcus* sp. and a *Pseudomonas* sp. Biodegradation measured by microbial growth, was reduced most by zinc and least by manganese.

There have been reports on metal stimulation of bacterial biodegradation processes under favourable environmental conditions of pH, temperature and aerobiosis [9], but Sandrin and Maier [10] observed that such stimulatory effects of metals on biodegradation occurred only when consortia, but not single microbial cultures were used for degradation processes. They argued that stimulation was a result of differential toxicity effects of the tested metals.

The manipulation of pH (which curiously has not been well studied) has been suggested as a possible approach to reducing heavy metal toxicity to hydrocarbon-degrading microorganisms [10]. This research reports on the influence of pH on the response patterns of fungal isolates to varying concentrations of cadmium and copper during biodegradation of spent engine oil.

Materials and methods

Collection of soil samples

Soil samples were collected from 3 automobile workshops at Mgbuka-Nkpor (6^o9’N 6^o50’E), Nigeria, using the method described in Mbachu *et al*. [11, 12]. The hydrocarbon (SEO) used in this work was subsequently collected direct from the engine of 911 Lorry (at Mgbuka-Nkpor) in a sterile container. Samples were transported to the laboratory for analysis.

Isolation of fungi with spent engine oil utilizing abilities

Fungi were isolated from soil samples obtained from spent engine oil polluted soil on Mineral Salt agar Medium using the method described in Mbachu *et al*. [13]. Each distinct colony on oil degrading enumeration plates were purified by repeated sub culturing onto Sabouraud Dextrose Agar (SDA) (Merck, Germany) plates. The pure isolates were maintained on SDA slant.

Identification of the isolates

The cultural characteristics of the pure isolates on SDA were noted, and the microscopic features were observed using the wet mount and the micro slide culture technique with reference to the Manual of Fungal Atlas [14, 15]. The isolates were also confirmed using 18S rRNA gene sequence.

Phylogenetic analysis

A phylogenetic tree was constructed using the neighbor-joining method, in MEGA version 4.1 [16]. The topology of the distance tree was tested by resampling data with 1000 bootstraps to provide confidence estimates.

Heavy metal characteristics of spent and new engine oil

Heavy metal analysis of spent and new engine oil was conducted using Varian AA240 Atomic Absorption Spectrophotometer [17].

Effect of varying concentrations of heavy metals on SEO degradation at different pH levels

The heavy metal salts employed in this study include: cadmium chloride ($CdCl_2$) and copper (II) tetraoxosulphate (VI) ($CuSO_4$). A weight of each of these metal salts that gave a corresponding 1g of each of the respective metal was weighed and dissolved in 1000 ml of sterile deionized water. These were left to stand for 30 minutes to ensure complete dissolution. Working concentrations of 0.1, 1.0, 10, and 100mgL^{-1} of the respective metals were prepared by serial dilutions of the stock solution [18] in mineral salt broth.

The effect of varying concentrations of Cd on SEO degradation at different pH was determined as described in Ekpeyong and Antai [19]. Tubes of mineral salt broth (MSB) (10ml) supplemented with 500 µl of SEO as carbon source and different concentrations of cadmium which included 0.1, 1, 10 and 100 mgL^{-1} were prepared, and the pH adjusted to 4.5, 5.5 and 6.5 levels, using concentrated orthophosphoric acid and 1M NaOH. The tubes were then inoculated with the pure as well as the mixed culture of the two isolates; *Candida tropicalis* and *Aspergillus clavatus* and incubated in triplicate in an orbital shaker at 120rpm at ambient temperature of 28^{o}C for 16 days. Control tubes which included: control 1 (un-inoculated); MSB + SEO without inoculum and heavy metal, control 2 (inoculated); MSB + SEO + Inoculum without heavy metal, were also set up.

The residual hydrocarbon remaining in the tubes after 16 days was determined by Spectrophotometric method [20], and compared with the control. The effect of heavy metals on spent engine oil degradation, expressed as percentage inhibition [5] was determined using the formula;
% Inhibition = concentration of residual oil in test sample – concentration of residual oil in control 2/concentration of residual oil in test sample X 100.

The growth response of the isolates during utilization of spent engine oil in the presence of varying concentrations of Cd at different pH was also determined by measuring the fungal biomass at 540 nm [21]. The fungal cells were harvested by centrifugation (3000 rpm for 30 minutes), washed twice with phosphate buffer and resuspended in 5 ml phosphate buffer. The absorbance was then measured at 540 nm. The whole process was also repeated for copper.

Results

Isolation and identification of fungi with spent engine oil utilizing abilities

Two fungi isolates obtained from soil samples contaminated with spent engine oil were identified as yeast and mold, based on their cultural and microscopic characteristics. Database comparism using BLAST program revealed that the yeast isolate had a high similarity of 98 % with those of strain *Candida tropicalis* MCCC2E00325 while the mold isolate had 100% similarity with those of strain *Aspergillus clavatus* ATCC 1007. The expected values (E-value) for the isolates are zero.

Phylogenetic analysis

The phylogenetic relationships of the sequence of the ITS region of the isolates are shown in Figures 1 and 2. Based on the phylogenetic trees, the closest relatives of the yeast and mold isolates are respectively, *Candida tropicalis* and *Aspergillus clavatus*.

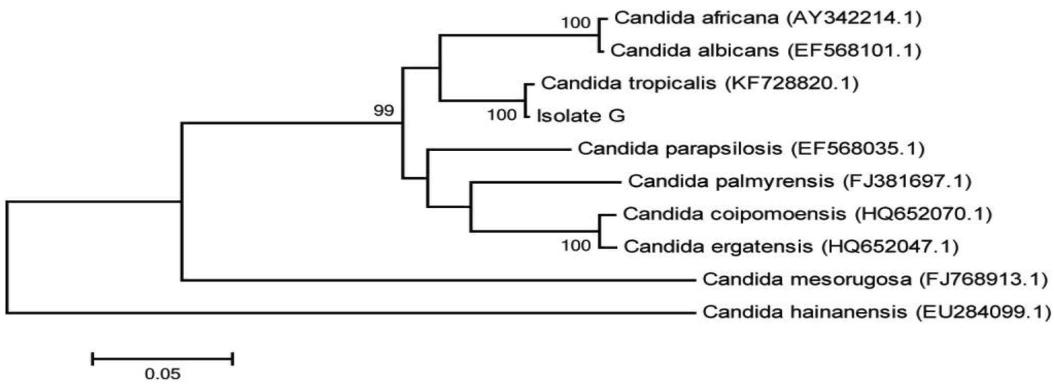

Fig. 1 Phylogenetic tree obtained by neighbor-joining analysis of the ITS sequences of the yeast isolate (*Candida tropicalis*). Support values for neighbor-joining were established by boostrapping with 1000 replicates, and the scale bar represents 0.05 sequence divergence. *Candida hainanensis* was used as the outgroup.

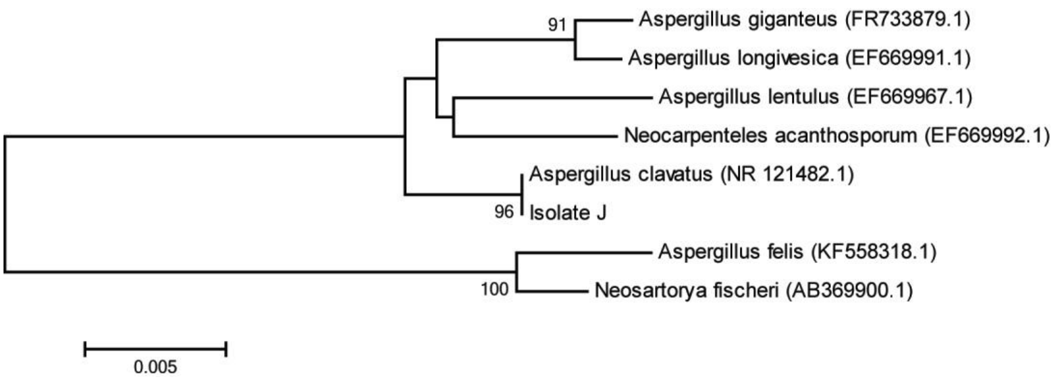

Fig. 2 Phylogenetic tree obtained by neighbor-joining analysis of the ITS sequences of the mold isolate (*Aspergillus clavatus*). Support values for neighbor-joining were established by boostrapping with 1000 replicates, and the scale bar represents 0.005 sequence divergence. The outgroup we used was *Neosartorya fischeri*.

Heavy metal characteristics of spent and new engine oil

The result of heavy metal analysis of spent and new engine oil is presented in Table 1. Analysis of variance (ANOVA) shows that the concentration of the heavy metals (Zn, Pb, Cd and Cu) was significantly higher ($P < 0.05$) in spent engine oil when compared to the new engine oil. However, Zn was predominantly found in spent engine oil (Table 1).

Table 1 Heavy metal analysis of spent and new engine oil

Samples	Zinc (ppm)	Lead (ppm)	Cadmium (ppm)	Copper (ppm)
Spent engine oil	7.593±0.007^{a}	0.040±0.001^{a}	0.106±0.008^{a}	0.119±0.004^{a}
New engine oil	1.877±0.026^{a}	0.008±0.002^{a}	0.011±0.002^{a}	0.018±0.026^{a}

Values are mean of three replicates ± standard deviation (SD).
Comparism of means along the columns; values followed by letter 'a' indicate significant difference at $P < 0.05$.

Effect of varying concentrations of heavy metals on SEO degradation at different pH levels

The effects of varying concentrations of cadmium on spent engine oil degradation at different pH levels are presented in Figures 3 to 5. In the media containing *C. tropicalis*, there was negative inhibition (stimulation) in spent engine oil degradation in the presence of 0.1 to 10 mg/l Cd at pH 5.5. However, maximum stimulation was recorded in the presence of 0.1 mg/l cadmium. An increasing trend in inhibition of spent engine oil degradation was observed in the presence of 0.1 to 100 mg/l cadmium at pH 4.5 and 6.5 (Fig. 3). Similarly, the OD was higher in the presence of 0.1 to 10 mg/l Cd, at pH 5.5, when compared to the control (Fig. 3). However, maximum OD was recorded in the presence of 0.1 mg/l Cd at pH 5.5. At pH 4.5 and 6.5, a decreasing trend in OD was observed as the Cd concentration increased from 0.1 to 100 mg/l (Fig. 3).

There was also stimulation in spent engine oil degradation in the media containing *A. clavatus*, in the presence of 0.1 to 10 mg/l Cd at pH 5.5, and maximum stimulation was achieved in the presence of 0.1 mg/l Cd. At pH 4.5 and 5.5, there was also an increasing trend in inhibition of spent engine oil degradation in the presence of 0.1 to 100 mg/l Cd (Fig. 4). Similarly, the OD was higher at pH 5.5, in the presence of 0.1 to 10 mg/l Cd, when compared to the control. However, at pH 4.5 and 6.5, there was a decreasing trend in OD as the Cd concentration increased from 0.1 to 100 mg/l (Fig. 4).

In the media containing the mixed culture of the isolates, there was stimulation in spent engine oil degradation in the presence of 0.1 to 1.0 mg/l Cd at pH 5.5, while an increasing trend in inhibition of spent engine oil degradation was observed in the presence of 0.1 to 100 mg/l at pH 4.5 and 6.5 (Fig. 5). Similarly, the OD was higher in the presence of 0.1 to 1.0 mg/l Cd when compared to the control, at pH 5.5. Moreover, a decreasing trend in OD was also observed as the Cd concentration increased from 0.1 to 100 mg/l at pH 4.5 and 6.5 (Fig. 5).

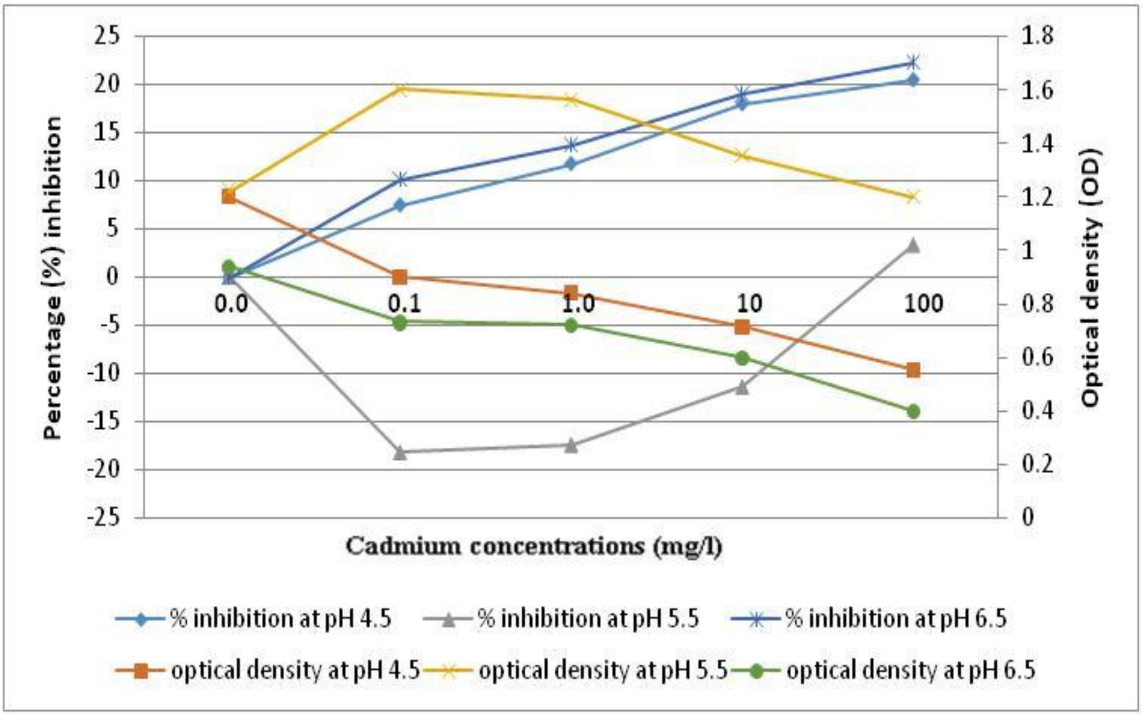

Fig. 3 Effect of varying concentrations of cadmium on SEO degradation by *C. tropicalis* at different pH.

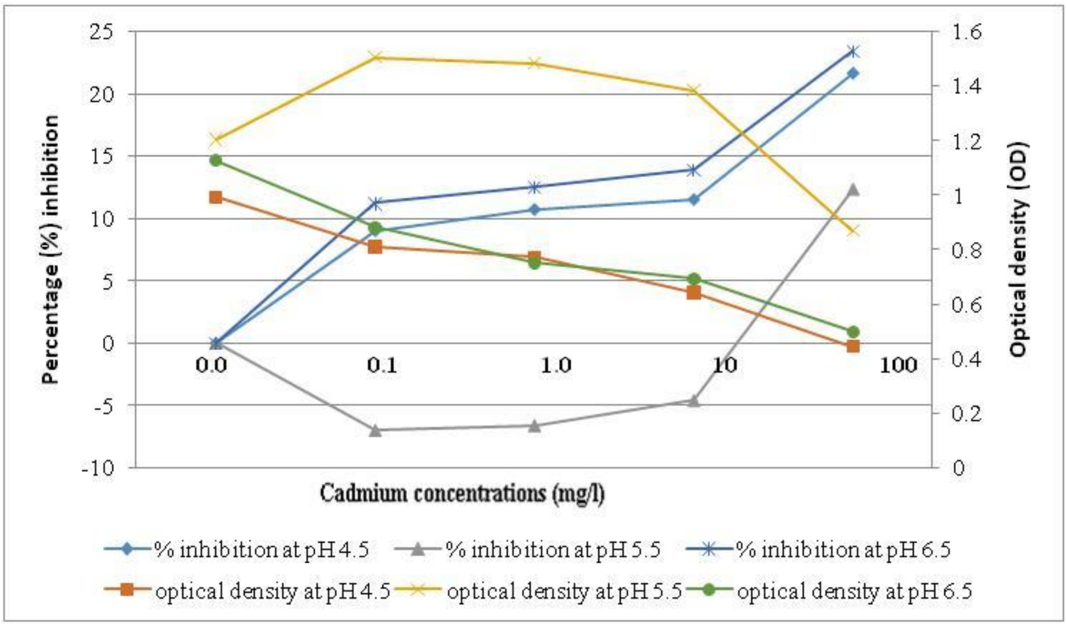

Fig. 4 Effect of varying concentrations of cadmium on SEO degradation by *A. clavatus* at different pH.

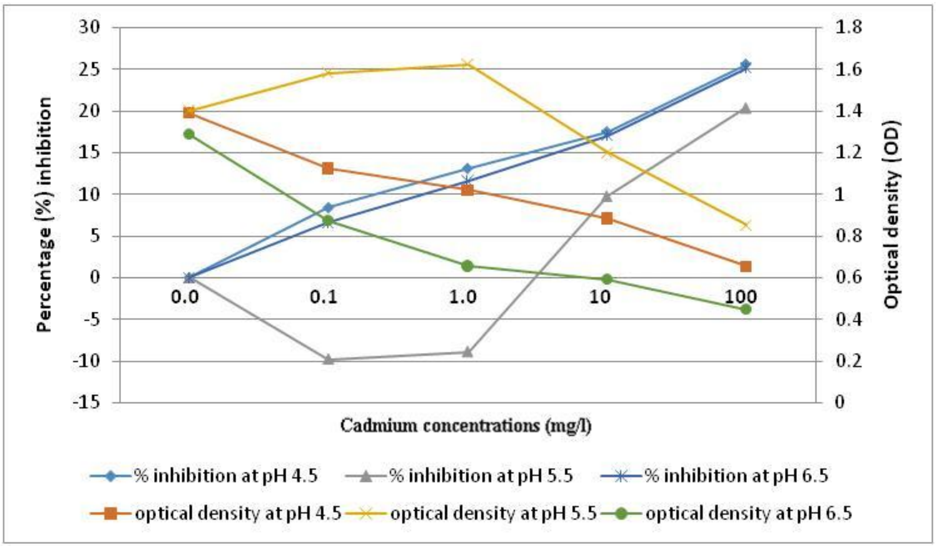

Fig. 5 Effect of varying concentrations of cadmium on SEO degradation by mixed culture at different pH.

The effects of varying concentrations of copper on spent engine oil degradation at different pH levels are presented in Figures 6 to 8. There was an increasing trend in stimulation of spent engine oil degradation in the media containing *C. tropicalis*, in the presence of 0.1 to 10 mg/l Cu at pH 5.5. A stimulatory effect on spent engine oil degradation was also observed in the presence of 0.1 mg/l Cu at pH 4.5. However, at pH 6.5, there was an increasing trend in inhibition of used engine oil degradation in the presence of 0.1 to 100 mg/l Cu (Fig. 6). The optical density was higher in the presence of 0.1 to 10 mg/l Cu, when compared to the control, at pH 5.5 (Fig. 6). The OD was also higher in the presence of 0.1 mg/l Cu at pH 4.5, when compared to the control. At pH 6.5, there was a decreasing trend in OD in the presence of 0.1 to 100 mg/l Cu (Fig. 6).

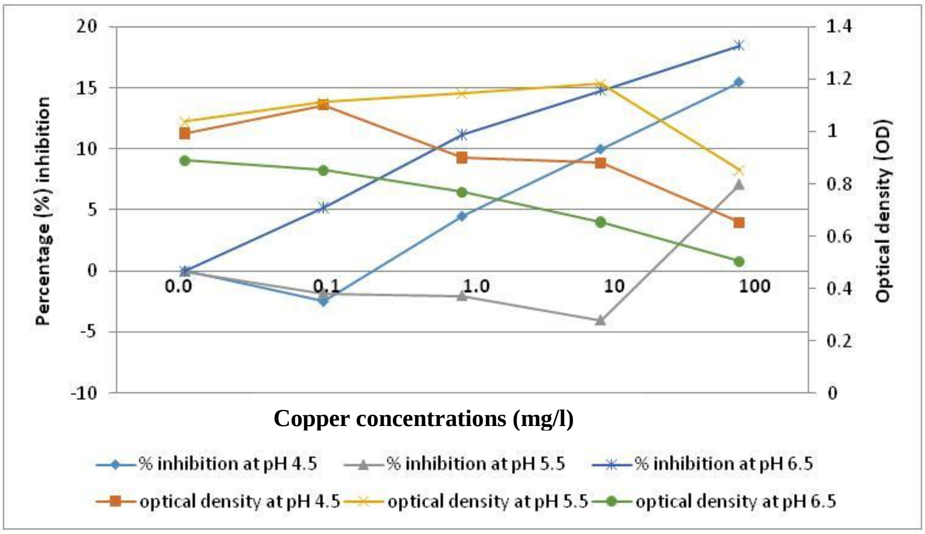

Fig. 6 Effect of varying concentrations of copper on SEO degradation by *C. tropicalis* at different pH.

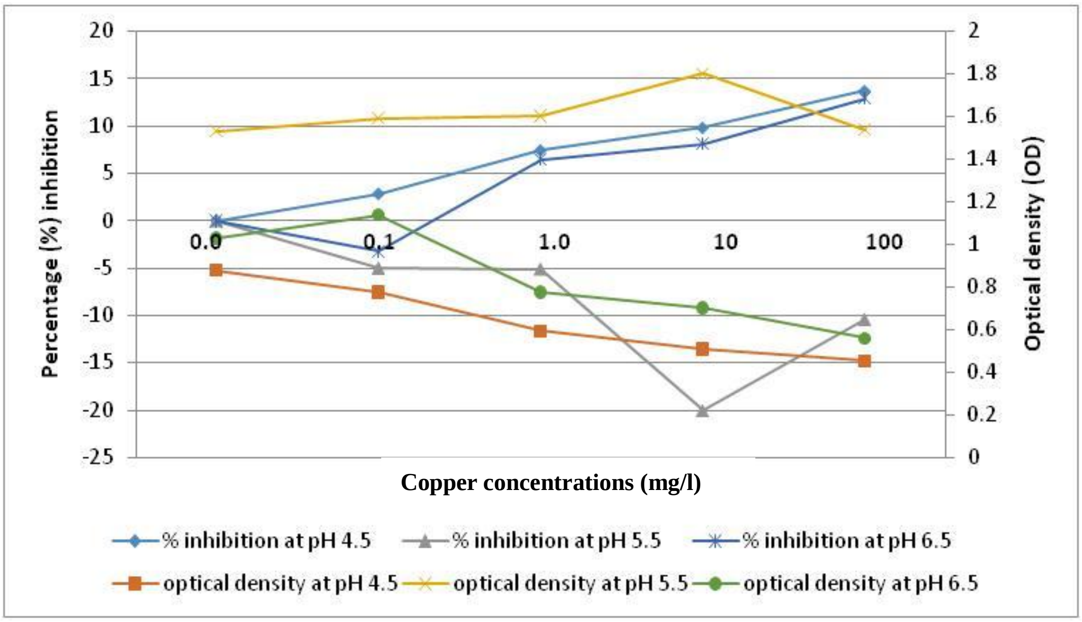

Fig. 7 Effect of varying concentrations of copper on SEO degradation by *A. clavatus* at different pH.

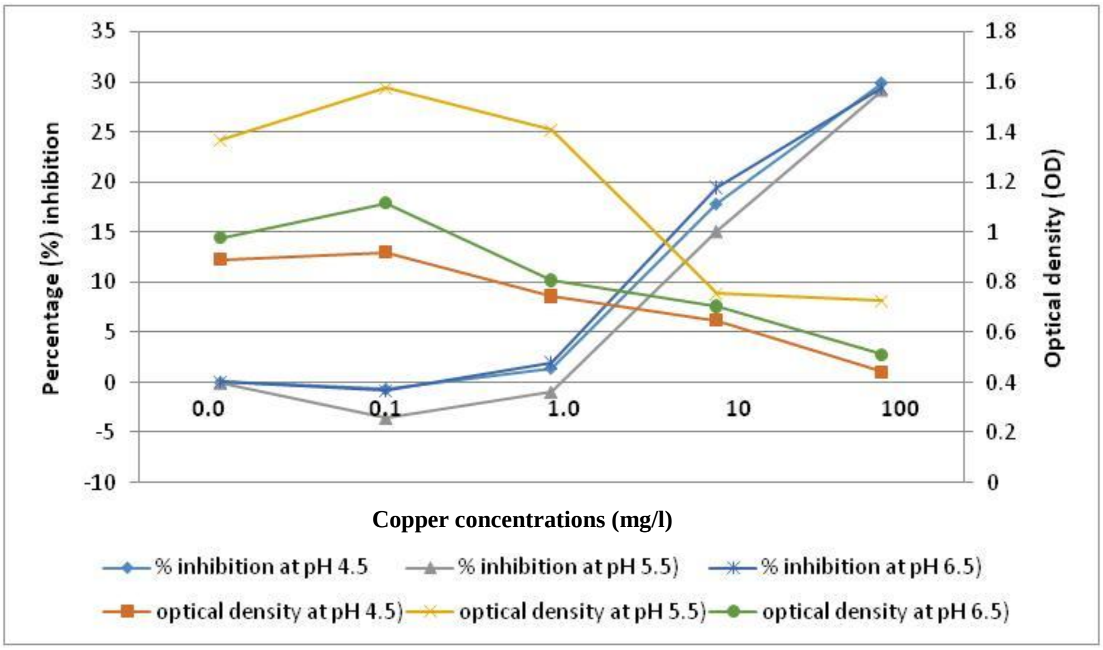

Fig. 8 Effect of varying concentrations of copper on SEO degradation by mixed culture at different pH.

In the media containing *A. clavatus*, there was stimulation in spent engine oil degradation in the presence of 0.1 to 100 mg/l Cu at pH 5.5. However, highest stimulation was recorded at 10 mg/l Cu at pH 5.5. At pH 6.5, there was also stimulation in spent engine oil degradation in the presence of 0.1 mg/l Cu, while at pH 4.5, an increasing trend in inhibition of spent engine oil degradation was observed in the presence of 0.1 to 100 mg/l Cu (Fig. 7). Similarly, there was increase in OD in the presence of 0.1 to 100 mg/l Cu at pH 5.5, when compared to the control (Fig. 7). Highest OD was recorded at 10 mg/l Cu at pH 5.5. At pH 6.5, the OD was higher at 0.1 mg/l Cu, when compared to the control, while at pH 4.5, a decreasing trend in OD was observed in the presence of 0.1 to 100 mg/l Cu (Fig. 7).

In the media containing the mixed culture of the isolates, there was stimulation in spent engine oil degradation in the presence of 0.1 to 1.0 mg/l Cu at pH 5.5. There was also stimulation in spent engine oil degradation at pH 4.5 and 6.5 at 0.1 mg/l Cu, while inhibition increased as the Cu concentration increased from 1.0 to 100 mg/l (Fig. 8). The OD was higher in the presence of 0.1 to 1.0 mg/l Cu at pH 5.5, when compared to the control (Fig. 8). At pH 4.5 and 6.5, the OD was also higher in the presence of 0.1 mg/l Cu, when compared to the control, while a decreasing trend in OD was however observed as the Cu concentration increased from 1.0 to 100 mg/l (Fig. 8).

Discussion

The higher level of heavy metals (Zn, Pb, Cd and Cu) found in spent engine oil compared to new engine oil could be due to the use of the lubricant in car engine. As motor oil circulates through a car's engine, it picks up heavy metals such as arsenic, lead, cadmium, copper and zinc [22]. Haytham and Ibrahim [23] also demonstrated the presence of several heavy metals such as lead, nickel, manganese, copper and zinc in used engine oil.

Despite the report that Cd was among the most toxic metal to microorganisms [24], these isolates; *C. tropicalis* and *A. clavatus* were able to grow well in the presence of up to 10 mg/l Cd at pH 5.5. The stimulation of used engine oil degradation with corresponding increase in the growth of the organisms at high Cd concentration was similar to the findings of Ramasamy *et al*. [25], who reported maximum growth of *Aspergillus* sp. in the media containing 10 mg/l Cd. This may be attributed to the biosurfactant producing ability of the isolates. It is possible that biosurfactant secreted by the isolates acted as metal chelating agents, reducing the bioavailability of Cd and rendering the metal less toxic. Das *et al*. [26], reported that a biosurfactant produced by *Bacillus circulans* was able to chelate positively charged lead and cadmium to the outer hydrophilic surface of biosurfactant, which consisted of anionic peptide head groups.

The stimulation in used engine oil degradation observed in the presence of high concentrations of copper may be attributed to the source of isolation of the organisms. This observation was in agreement with the findings of Iram *et al*. [27], who reported that fungal populations isolated from heavy metal contaminated sites have the ability to resist higher concentrations of metals. However, the tolerance and the resistance of the isolates depended more on the fungus tested than on the site of its isolation, since all the isolates were obtained from the same source but exhibiting different levels of tolerance. This was evidenced by the higher level of tolerance to copper exhibited by *A. clavatus* in this study. Similar results were reported by Price *et al*. [28], who showed that *Aspergillus* was able to tolerate heavy metals as compared to other fungi. The most probable reason for the difference in resistance levels could be the variation in the mechanism of resistance [29, 30]. This variation may be explained by the development of tolerance or adaptation of the fungi to heavy metals.

Although high metal concentrations inhibit pollutant biodegradation, the study revealed that pH 5.5 had positive influence in the biodegradation of spent engine oil co-contaminated with heavy metals (cadmium and copper) by the pure and mixed culture of the isolates *Candida tropicalis* and *Aspergillus clavatus*. The pure and mixed culture of the isolates (*Candida tropicalis* and *Aspergillus clavatus*) could therefore be utilized in the bioremediation of spent engine oil contaminated soil co-contaminated with heavy metals, at appropriate pH levels.

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