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(RESEARCH ARTICLE)

The impact of concentration on the biotreatment of hair relaxers using Hydrocarbonoclastic *Pseudomonas* and *Mucor Species* from Ezu River, Awka, Nigeria

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Abstract

The contamination of an aquatic environment by hydrocarbons and other noxious compounds due to human activities may cause toxicity to microorganisms and may stimulate microbial activity especially at low concentrations while their persistence can be detrimental to the entire ecosystem. It is crucial therefore, to assess the risks of these pollutants for environmental policy. Diesel was used as carbon source in mineral salts medium for the isolation of petroleum degrading microorganisms from Ezu River using vapor transfer technique. Plates were incubated at 37 ^{0}C and 28 ^{0}C for bacterial and fungal cultures, respectively. Of the 23 bacterial and 4 fungal isolates recovered, *Mucor species* and *Pseudomonas aeruginosa* were the best hydrocarbon utilizing isolates obtained. The isolates were assessed for their ability to utilize both lye-containing and lye-free hair relaxers at different concentrations (0.2 – 1% w/v) in mineral salts medium for 14 days. The optical density (OD660nm), the biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), and pH of the medium were monitored during incubation. The toxicity of the hair relaxers to *Nitrobacter sp*. was also assessed. Results showed that *Pseudomonas aeruginosa* utilized both lye-free(Lf) and lye-containing (Lc)hair relaxers at all concentrations as indicated by the high colony counts, reduction in BOD_5 and COD during the incubation period. *Mucor sp*. grew better on higher concentrations Lc hair relaxer (1%> 0.8%> 0.6% w/v), but with minimal growth at lower concentrations of same relaxer (0.2%< 0.4%< 0.6% w/v). On the contrary, lye-free hair relaxer supported heavy growth of *Mucor sp*. at all concentrations tested. The growth of *Nitrobacter sp*. got inhibited by both types of hair relaxers between concentrations 0.6 and 1% w/v, with moderate growth at lower concentrations (0.2 – 0.4% w/v). The toxicity outcome of these hair relaxers to *Nitrobacter sp*ecies implies that the indiscriminate discharge of hair-relaxer-containing salon wastewater into the soil may affect the biogeochemical activities of the soil microbial communities. In addition, *Pseudomonas aeruginosa* and *Mucor species* recovered in this study can be used to attenuate sites contaminated by hair salon wastewater.

Keywords: Biotreatment; Hydrocarbonoclastic; Hydrocarbon contamination; Attenuation

1. Introduction

Need for consortium of indigenous bacterial and fungal species with excellent option of introducing specific consortium of these microorganisms in a contaminated site during a bioremediation process in Nigeria and cannot be over emphasized since they are cost effective and yield positive results in a shorter time . The hydrocarbon-utilizing bacterial and fungal isolates from Ezu river were used to utilize both lye-containing (Lc) and lye- free (Lf) hair relaxers. The Lc hair relaxers are those that contain sodium hydroxide (NaOH) while the Lf hair relaxers are those relaxers that contain potassium hydroxide (KOH), lithium hydroxide (LiOH), alkaline perms (guanidine hydroxide) which is made of calcium hydroxide ($CaOH)_2$ and guanidine carbonate (Dias, 2015). Another form of Lf hair relaxer is the ammonium thioglycolate (perm salt). The contamination of both aquatic and terrestrial environments by hydrocarbons such as hair relaxers may cause toxicity to microorganisms while some may stimulate microbial activity especially at low concentrations (Odokuma *et al.,* 2015*).* The persistence of some contaminants in the environment affect human health and the

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ecosystem, hence it is crucial to assess the risks of these pollutants for environmental policy. The determination of precise concentration effect relationships can be restricted to the most sensitive organisms *(*Kekacs *et al.* 2015*)*. The hydrocarbon- utilizing isolates which gave impressive results in the degradation of diesel were used to degrade both Lc and Lf hair relaxers in a 14-day bioremediation process with the optimum time of utilization being between the 4th and 8th days as concentration increased (1% >0.8%>0.6%>0.4%>0.2%). *Pseudomonas and Mucor spp*. showed satisfactory utilization of different concentrations of both hair relaxers at all concentrations. Xenobiotics generally may affect the microorganisms' physiological process thus, when these relaxers get into soil and water environments through wastewater, they constitute toxicity problems. They can also affect the genetic machinery of the organisms living in the ecosystem and may cause inhibition of some important microbial communities (Gianfreda et al. 2017) and alter the physicochemical properties of the soil environment due to the nature of their composition (Alrymman *et al.* 2015). The aim of this study is to use hydrocarbonoclastic bacterial and fungal species isolated from Ezu river to degrade varying concentrations of both Lye-containing and Lye-free hair relaxers as well as examine their toxicity to soil indigenous microorganisms using *Nitrobacter sp.* as model.

2. Material and methods

All the reagents and media used in this study were of analytical grade while the hair relaxers were purchased locally from a department store at Awka. The hydrocarbon used in this study is diesel and was purchased from the Nigerian National Petroleum Corporation (NNPC) Mega Filling Station located in Awka, Anambra State.

2.1. Study Area

The water samples used were collected from Ezu river which is located along the Enugu Onitsha Expressway by Amansea town in Awka North Local Government Area of Anambra State. The river covers a reasonable area in both East and West sides of Enugu and Anambra State; and geographically serves as a boundary between the two States.

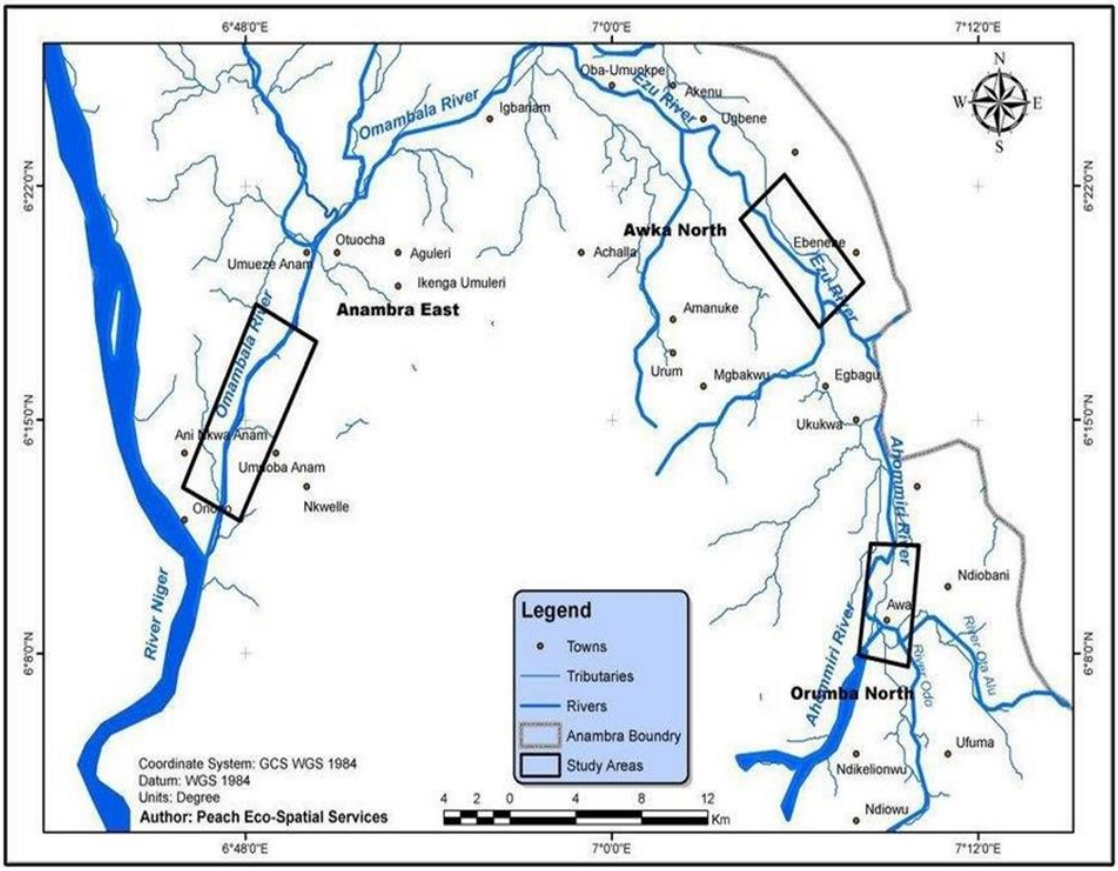

Figure 1 Map of Anambra State, Nigeria, showing the Sampling Location

2.2. Sample Collection

The samples of water were collected in four replicates at different points which included upstream (about 120m from the midstream), midstream and downstream (about 115m away from the midstream). The samples were collected 25cm from the riverbank and 6cm deep using sterile screw-capped specimen bottles held at the bottom and immersed into the water while setting the mouth at angle 45^0 against the direction of water flow and immediately taken to the laboratory in an ice box to preserve the integrity of the samples.

2.2.1. Enumeration of Microorganisms

Standard plate count technique was used to evaluate microbial populations in the samples. Total heterotrophic bacterial counts were carried out on nutrient agar plates while that of heterotrophic fungi was done using Sabouraud Dextrose Agar (SDA) plates, while the population of hydrocarbon-utilizers was evaluated by spread-plate technique on mineral salts (MS) medium as described by Mills *et al.* (1978) which was solidified using bacteriological agar, while diesel was supplied through vapour phase transfer as described by Chen *et al.* (2015). Plates were incubated at 35^oC for 7 days and the counts recorded as colony forming units per mill (cfu/ml).

2.3. Screening of Hydrocarbon – Utilizers

Discrete colonies obtained from mineral salts (MS) plates above were purified and further screened for the utilization of hydrocarbon using 1.0% (v/v) diesel and incubated into MS broth at 200 rev/min and at 35^oC for 5 days. Optical density (OD) was estimated at 660nm using spectrophotometer. The isolates were further examined for the utilization of different concentrations of both Lc and Lf relaxers by incubating at 200 rev/min for 14 days while observing some parameters such as optical density (OD), Biological Oxygen Demand (BOD_5) and Chemical Oxygen Demand(COD).

2.4. Identification of Hydrocarbon -utilizing Isolates

The heterotrophic bacteria were identified using keys provided in the Bergey's Manual of Systematic Bacteriology. The hydrocarbon-utilizing bacterial isolates were examined for both morphological and biochemical characteristics while the fungal isolates were examined both morphologically and microscopically using wet mount method while their identification was performed using a fungal atlas by Tsuneo (2000).

2.5. Isolation of *Nitrobacter species* and toxicity test

Nitrobacter sp. was isolated from the soil using the Winogradsky medium and nutrient agar base by spread plate technique. The standard toxicity procedure was applied using varying concentrations of both relaxers at % 0.2< 0.4< 0.6< 0.8< 1.0 and incubated for a period of 3days at room temperature.

3. Results

All the bacterial and fungal isolates capable of degrading diesel responded differently to varying concentrations of both Lye containing (Lc) and Lye-free (Lf) hair relaxers which served as sources of carbon and energy. Table 1 shows the Statistical results for the growth of some bacterial species with diesel as carbon and energy source. *Pseudomonas sp.* emerged as the ultimate diesel degrader with a high OD of 0.897 and mean value of 3.876 while for fungal isolates as shown in Table 2, *Mucor sp*. was the best utilizer with the highest biomass value of 1.66g. In the Evaluation of the COD of the mineral salt medium containing either Lc or Lf using *Pseudomonas sp.* as shown in Table 3, the COD increased as the concentrations of both relaxers increased (1%>0.8>0.6>0.4>0.2) likewise *Mucor sp.* as shown in Table 4 while the effect of different concentrations of both Lc and Lf relaxers on BOD using *Pseudomonas sp.*as a test organism is as illustrated in figures 2&3. The BOD increased as the concentration increased but decreased as the time (days) increased. The effect of concentrations of both Lc and Lf relaxers on BOD using *Mucor sp*. as the test organism is as shown in figs. 4 and 5 respectively. The highest BOD value was attained on the 4^{th} day at higher concentrations (1.0 > 0.8% 0.6>0.4>0.2%). However, the HC-utilizing fungal isolates were either partly stimulated or inhibited at different concentrations of the Lf except for *sp.* which had remarkable growth at all concentrations.

The growth of all the hydrocarbon (HC)-utilizing fungal isolates on both Lc and Lf relaxers are as shown in tables 3 and 4, respectively. While *Mucor* has been observed to be highly stimulated at all concentrations of both hair relaxers, LR totally inhibited the rest of the fungal isolates at all concentrations. However, the HC-utilizing fungal isolates were either partly stimulated or partly inhibited at either higher or lower concentrations of the Lf relaxer except for Mucor which had remarkable growth at all concentrations. *Pseudomonas* and *Mucor sp.* were selected as the prospective organisms for the bioremediation of both Lye (Lc) and Lye-free (Lf) hair relaxers and were engaged in a 14-day bioremediation process in a mineral salt broth during which, some parameters such as the Chemical Oxygen Demand (COD), Biological

Oxygen Demand (BOD), Optical density (OD) and pH were monitored for 14 days at 4days interval. The results are as represented in figures 2-7.

The COD obtained few hours after the inoculation of the samples with both types of hair relaxers showed that at higher concentrations, the COD increased but decreased at lower concentrations for both *Pseudomonas* and *Mucor* spp.

However, when the toxicity of both Lye and Lye-free relaxers on *Nitrobacter sp.* was assessed, it was observed that *Nitrobacter* grew heavily at lower concentration of 0.2%, moderately at 0.4%; and minimally at 0.6% but had no growth 0.8% and 1.0% of both types of hair relaxers which implies that there was inhibition as the concentrations increased.

Table 1 Statistical results for the growth of some bacterial species in mineral salts medium containing diesel as carbon source.

Isolate	OD		pH		Cfu/ml	
	Mean	SD	Mean	SD	Mean	SD
Pseudomonas aeruginosa	3.876	0.897	8.072	0.2949	3.46	0.6189
Bacillus sp.	3.838	0.4905	7.874	0.1101	2.700	1.046
Micrococcus luteus	3.100	0.6284	7.898	0.07823	2.000	0.9798
Corynebacterium sp.	2.636	0.8661	7.79	1.3766	1.720	0.7662
Flavobacterium sp.	2.316	0.2212	7.884	1.1459	1.870	0.5628
Klebsiella aerogenes	1.618	0.4717	7.904	0.0551	1.862	0.7701

OD = Optical density at 660mm; SD = Standard deviation

Table 2 The growth of fungal isolates in mineral salts medium containing diesel as carbon source.

Isolate	Initial p^H	Final p^H	Biomass(g)
Mucor sp.	7.4	7.08	1.66
Aspergillus fumigatus	7.4	7.15	0.93
Aspergillus flavus	7.4	7.12	0.65
Fusarium sp.	7.4	7.14	1.02
Penicillium sp.	7.4	7.1	0.82

Table 3 Evaluation of the COD of mineral salts medium containing either Lc or Lf with *Pseudomonas aeruginosa* as the test organism.

Concentration (%)	COD for Lc ($x10^2$mg/l)	COD_0 for Lf ($x10^2$mg/l)
1.0	14.65	3.15
0.8	11.99	2.79
0.6	10.21	2.44
0.4	04.0	2.35
0.2	0.44	1.47

Table 4 Evaluation of the COD of mineral salts medium containing either Lf or Lc with Mucor sp. as the test organism.

Concentration (%)	COD for Lc (x10²mg/l)	COD for LF (x10²mg/l)
1.0	9.95	3.24
0.8	1.91	3.24
0.6	0.93	2.71
0.4	0.49	1.55
0.2	0.04	0.04

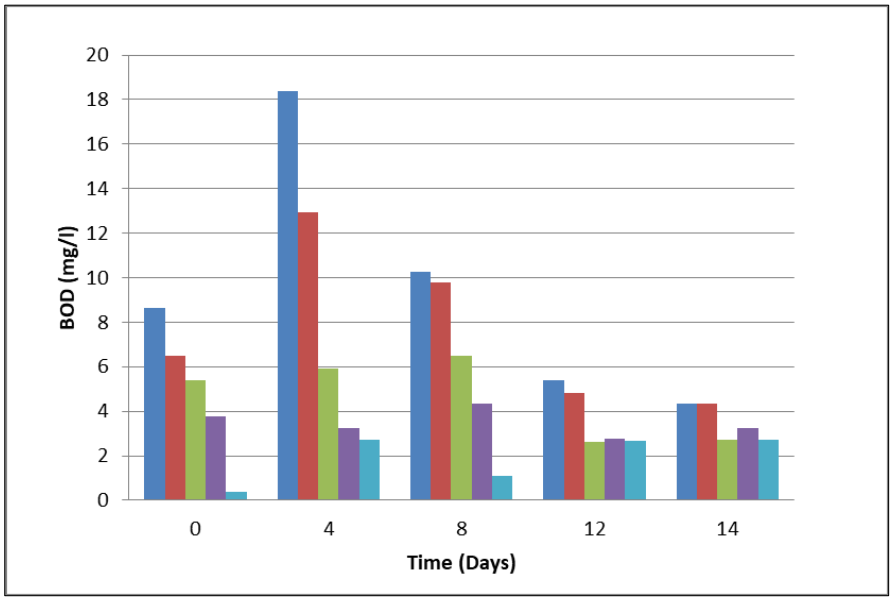

Figure 2 Effect of concentration of Lc on BOD (mg/l) during bioremediation using *Pseudomonas aeruginosa* as the test organism.

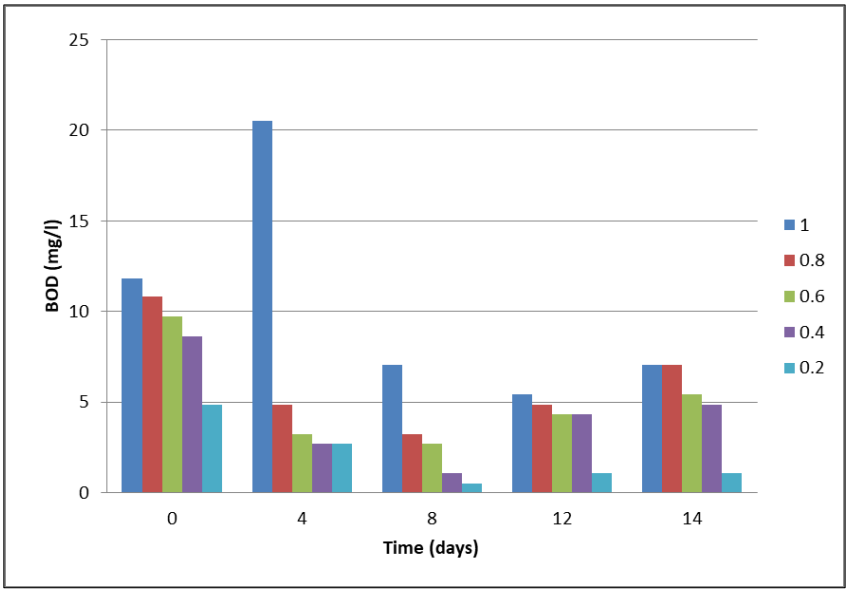

Figure 3 Effect of concentration of Lf on BOD (mg/l) during bioremediation using *P. aeruginosa* as the test organism

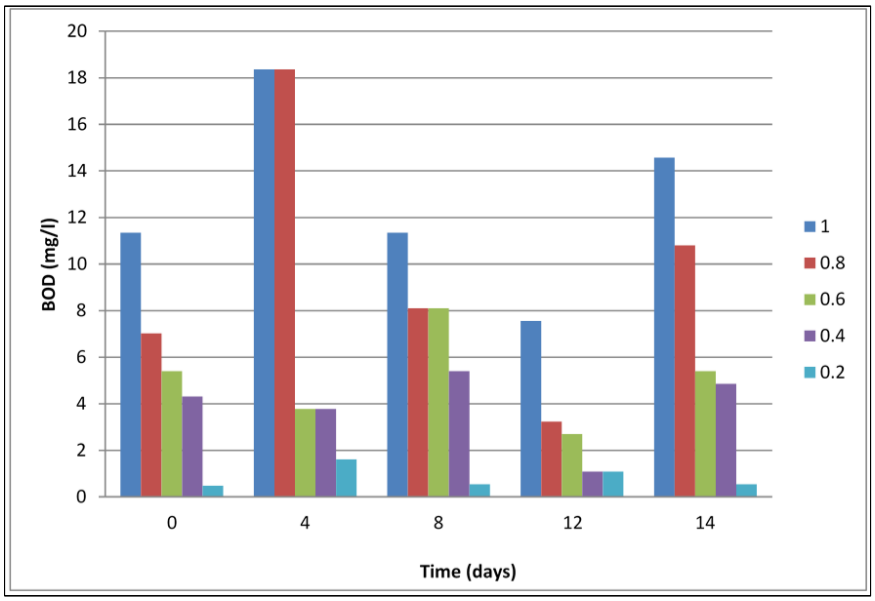

Figure 4 Effect of concentration of Lc on BOD (mg/l) during bioremediation using *Mucor* as the test organism

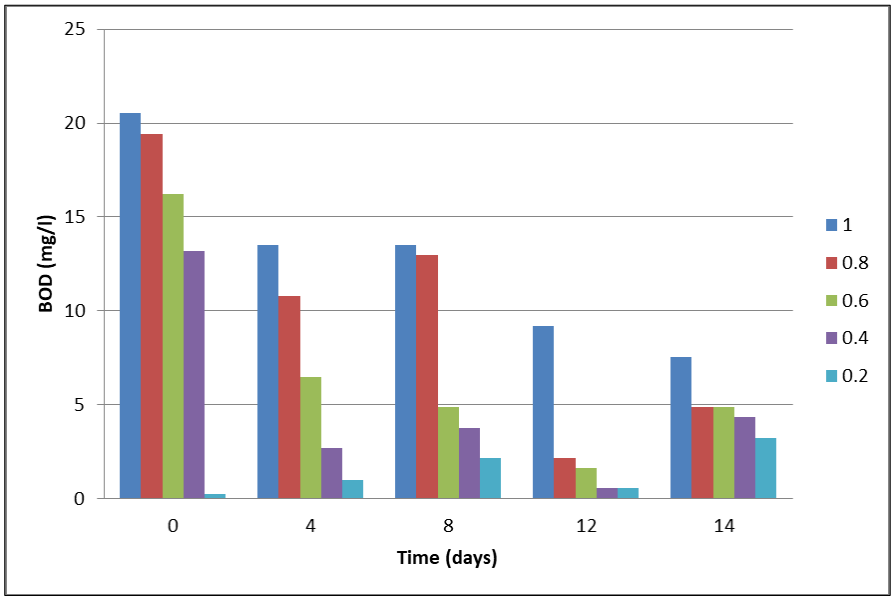

Figure 5 Effect of concentration of Lf on BOD (mg/l) during bioremediation using *Mucor sp.*as the test organism

4. Discussion

The Lye hair relaxer–utilizing fungal isolates include *Mucor*, *Aspergillus,* and *Penicillium species* while the bacterial isolates include *Pseudomonas, Micrococcus, Klebsiella, Corynebacterium,* and *Bacillus species.* On the other hand, the Lye-free hair relaxer– utilizing fungal isolates include *Mucor, Aspergillus, Fusarium and Penicillium spp.,* while bacterial isolates include *Pseudomonas and Bacillus species.*

The inhibition of *Aspergillus flavus, Fusarium* and *Flavobacterium spp*. by the L-containing hair relaxer at all concentrations and the stimulation on *A. fumigatus* and *Penicillium sp*. by Lye- free hair relaxers at different concentrations agrees with the report by Xiao(2015) that high concentrations of chemicals inhibited the growth of *Nitrobacter species* while low concentrations were stimulatory.

The response of the hydrocarbonoclastic isolates at varying concentrations of Lye-free and Lye-containing hair relaxers were studied and the results showed optimum population of *Pseudomonas* and *Mucor spp*. by the 12th day of the

bioremediation procedure which confirmed their logarithmic phases within the period and was indicative of their tolerance to varying concentrations of both relaxers tested on them.

The successful implementation of the use of specific bacteria and fungi species in the bioremediation of hair relaxers agrees with the respective reports made available by Mikolasch *et al.(*2019) and Badr El-Din (2014) that hydrocarbons are utilized by assorted number of microorganisms with emphasis on the fact that fungi and bacteria have catabolic abilities to degrade hydrocarbons. The application of *Pseudomonas sp*. in this study is in tandem with the report by Spini *et al* (2018) that during the isolation of bacteria and fungi species capable of degrading petroleum, the dominant bacteria during the process was *Pseudomonas sp.*

The value of the biological oxygen demand (BOD) attained by the 4th day of bioremediation of hair relaxer-contaminated waters was observed to have increased as the various concentrations decreased (1.0>0.80>0.6>0.4>0.2%). This agrees with the report by Rodríguez-Uribe *et al.* (2021) that hydrocarbon-utilizing *Pseudomonas sp.* from saline wetland soils contaminated with weathered petroleum oil has the capacity for the bioremediation of petroleum oil-contaminated soils and also agrees with the findings by Xu *et al*.(2020) that the increase in the bacterial population may be due to the stimulatory effect of the additional carbon and energy sources (in this case, hair relaxers)which led to the enrichment of the hydrocarbonoclastic microbial population as a result of their individual requirements for both carbon and energy sources.

The relaxers showed pronounced effect on the microbial population especially on *Nitrobacter sp*. present in the environment at higher concentrations but stimulatory at lower concentrations which agrees with the report by Xiao(2015) that *Nitrobacter* et al. *species* may serve as indicator for toxicity in wastewater emphasizing that high concentrations of chemicals inhibit the growth of Nitrobacter while low concentrations are stimulatory.

Furthermore, organisms such as *Mucor, Pseudomonas* and other bacteria and fungi species were able to utilize both relaxers. The growth of the microbial isolates on varying concentrations of Lye and Lye- free hair relaxers as the only source of carbon agrees with the findings of Premnath *et al*.(2021) that Microbial degradation of hydrocarbons is done by diverse microorganisms like bacteria and fungi while the rate of degradation by microbes is mainly governed by various cultivation conditions such as temperature, pH, nutrients availability and microbial population.

The Chemical Oxygen Demand (COD) values for both *Pseudomonas and Mucor spp.* obtained a few hours after inoculation of the relaxers increased as the concentration of both relaxers increased. This agrees with the report by Poorsoleiman *et al.*(2021) that the inoculation of microorganisms can accelerate the initial phase of bioremediation in some cases. This also agrees with the report by Prakash *et al.(*2015) that microbial activities allowed the mineralization of some petroleum components into CO_2 and water, stating that microbial transformation is a major route for complete degradation of petroleum components.

A study of the physical parameters monitored during the bioremediation procedure revealed that the temperature(^{0}C) was fluctuating due to different response patterns of the individual microorganisms to stimulus. The pH and Optical Density(OD) increased as the concentrations increased due to the chemical constituents of the relaxers. This agrees with the work of Zhang *et al*. (2017) that there could be changes in the pH values and temperature of the culture medium depending on the composition of the substrate.

During the determination of fungal growth using different concentrations of both Lye-containing and Lye-free hair relaxers for biomass estimation, the substrates were utilized for cell growth. This agrees with the work of Krithika and Philip (2016) who reported that the rates of substrate degradation are linked to the rates of growth in a manner that the concentration of the biomass increases with substrate depletion.

The stimulatory effect of both hair relaxers on *P. aeruginosa* and *Mucor sp*. at low concentrations during this bioremediation process was confirmed by the increase in biomass throughout the experimental period. They therefore have great potential in facilitating the remediation of hydrocarbon-polluted water. This finding agrees with that of Asemoloye *et al.* (2020) that *Mucor sp*. has the potential to be used in the biotreatment and removal of hydrocarbons from polluted soils.

5. Conclusion

This study has proven that the inoculation of microorganisms can accelerate the initial phase of bioremediation in some cases of site contamination by hydrocarbons. However, microorganisms such as *Pseudomonas and Mucor spp.* recovered during this study, can specifically be used to attenuate sites contaminated by both lye and lye free hair relaxers

contained in salon wastewater discharged into the environment. The toxicity to *Nitrobacter* at high concentrations with stimulatory effect at lower concentrations implies that the growth of some aquatic and terrestrial microorganisms may be enhanced, inhibited, or adversely affected by salon wastewater containing any of these hair relaxers and may affect important agricultural microorganisms present in the plant rhizosphere, thereby, determining the productivity of such plants.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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