BIODEGRADATION OF TREATED REFINERY WASTEWATER USING MUTANT STRAINS OF SELECTED MICROBIAL SPECIES

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ABSTRACT: Due to high cost of treatment technologies, petroleum industries discharge their wastewater (effluent) without effective treatment, thereby causing pollution. This research was done to determine the microbial and physicochemical parameters of treated effluent of Warri petrochemical company and the petroleum degradability of modified *Bacillus* and *Pseudomonas* species on the effluent. Isolates from refinery effluent were determined using standard bacteriological methods and modified using x-ray mutagen. Microbial degradation of total petroleum hydrocarbon (TPH) in the refinery effluent revealed that *Pseudomonas sp* had a higher degradation potential than *Bacillus sp*. Greater reduction in biochemical oxygen demand (BOD) values was observed in the modified strain of isolates than their parents. TPH values for *Bacillus* + Effluent reduced from 0.98 to 0.26 and 0.98 to 0.18 for *Bacillus* + Effluent +fertilizer. Mutant *Bacillus*+ Effluent and Mutant *Bacillus*+Effluent+fertilizer reduced from 0.98 to 0.25 and 0.22 respectively. Similarly, TPH values for *Pseudomonas* + Effluent and *Pseudomonas* +Effluent +fertilizer reduced from 0.98 to 0.11 and 0.04 respectively. Mutant *Pseudomonas* + Effluent and Mutant *Pseudomonas* +Effluent +fertilizer reduced from 0.98 to 0.12 and 0.22 while the consortium of organisms +Effluent +fertilizer and control reduced from 0.98 to 0.20 and 0.67 respectively. The highest TPH degradation of 95.9% was observed for *Pseudomonas* +Effluent +fertilizer while the least (73.5%) was observed for *Bacillus* + Effluent. Statistical analysis of variance at 95% confidence level indicates that there was significant difference between the bioremediation potentials of both parents and their mutants. Therefore, *Pseudomonas species* and their mutants should be employed in bioremediation of petroleum hydrocarbon wastewater.

Keywords: *Bacillus, Pseudomonas*, mutant, degradation, microorganisms, effluent.

Introduction

Environmental pollution by petroleum and petrochemical products has been a major challenge in Nigeria (Maheen *et al.*, 2022). This pollution results from increase in petroleum exploration, refining and other allied industrial activities (Maheen *et al.*, 2022). Large quantities of polycyclic aromatic hydrocarbons, heavy metals, inorganic salts, phenols, metal derivatives, surface active substances, sulfides, naphthylenic acids and other chemicals present in wastewater come from petroleum refining and petrochemical company (Ajao *et al.*, 2022). Accidental discharge of crude petroleum, field oil and grease from the refinery also contribute to pollution (Emoyan *et al*., 2021). Due to ineffective treatment of the wastewater (effluent), the pollutants find their way into receiving water bodies thereby posing serious threat to aquatic life and the ecosystem in general (Ajao *et al.*, 2022). Crude oil causes serious damage to humans and the ecosystem (Mace *et.al* 2020). The mortality rate of water related diseases according to World health organization exceeds five million people annually with microbial intestinal infections accounting for more than 50% (Cabral, 2010). However, natural biological activities can be adopted to render various contaminants harmless. In addition to the high cost, the conventional cleaning technologies used for remediation of refinery effluent do not lead to complete degradation of pollutants (Chukuka *et al*., 2023). The need arises therefore for an environmentally friendly and cost effective cleaning technology (Ojo *et al*., 2021, Hamza *et al.*, 2012). Bioremediation process uses microorganisms to breakdown harmful pollutants thereby transforming them into harmless products using simple, low cost techniques to clean up oil pollution. Bacteria, fungi and archea are often used in bioremediation processes to convert wastes to carbon dioxide, water and other products. Bioremediation is reliable, cheap and eco-friendly but depends on environmental factors such as nutrient availability, moisture content and temperature for maximum efficacy. The indigenous microbes are thus biostimulated to perform the bioremediation process (Hamza *et al*., 2012, Hamza *et al.*, 2023, Koshlaf and Bail, 2017). This study employed indigenous species of *Bacillus and Pseudomonas* to clean-up petroleum refinery wastewater by bioremediation. The research work was aimed at developing a cost effective technique for the treatment of refinery wastewater using modified microbes by determining the biodegradation potentials of indigenous but modified strains of *Bacillus and Pseudomonas* sp.

The objectives were to isolate *Bacillus* and *Pseudomonas sp.* from treated refinery effluent, use X-ray to modify the organism and determine the bioremediation potentials of the parent organisms and their modified strains.

Methodology

Collection of sample

All samples used for this research were obtained from Warri refining and petrochemical company. 10litres of wastewater (effluent) was taken at the point of its discharge into Ubeji creek and transported in ice chest to prevent growth of microorganisms, to the Microbiology laboratory of Delta State University, Abraka.

Physicochemical analysis of Effluent

The wastewater was analyzed for physicochemical, organic and heavy metal constituents at the start and end of the bioremediation period.

Biochemical oxygen demand (BOD) was determined in accordance with the method of ASTM designation D-6697-01.

Chemical oxygen demand (COD) was also determined in accordance with the method of ASTM designation D-6697-01.
Total dissolved solids (TDS) was determined following the procedures of ASTM designation D-989.
Total suspended solids (TSS) was determined following the procedures ASTM designation D-1909-00.
The turbidity of the sample was carried out using the method of ASTM designation D-1889-00.
The total solid (TS) was taken recorded as the summation of the results of total dissolved and total suspended solid.
Dissolved oxygen (DO) was determined using a Jenway DO meter probe by dipping it into the sample.
Temperature was determined using digital Jenway H thermometer of range 0-100^{o}C.
pH was determined using pH meter (model no 7020) respectively.
Electrical conductivity was done using the methods of ASTM designation D-1125-95.
Nitrate was determined using ASTM designation D-3867-99.
Alkalinity was determined using ASTM designation D-1990.
Sulphate was determined using ASTM designation D-4658-92 respectively.
A control experiment was set-up. This set- up was without any microorganisms.
All readings were taken in accordance to manufacturer's instruction

Isolation and Identification of Bacteria

Isolation of bacteria from the effluent was done under strict aseptic conditions using standard dilution methods. Nutrient agar plates inoculated with 0.1 ml of diluted sample were incubated at 37^{o}C. Slight growth was observed after 24 hours. However, samples were left for up to 72 hours (three days) after which distinct colonies were formed. Single colonies of bacteria were then sub-cultured into fresh nutrient agar plates and incubated at 37^{o}C for 48 hours. Morphological and biochemical identification of bacteria isolates was done in accordance with the method given in Bergey's manual of systematic bacteriology.

Assessment of potentials of microorganisms to degrade Hydrocarbon.

Pure colonies of isolates were inoculated in Bushnell-Haas medium (magnesium sulphate 0.2gm/l, calcium chloride 0.02gm/l, monopotassium phosphate 1.0gm/l, Dipotassium phosphate 1.0gm/l, ammonium nitrate 1.0gm/l, ferric chloride 0.05gm/l, agar 20.0gm/l, pH 7.0±0.2) and incubated at 37^{o}C for 72 hours. Appearance of microbial growth on culture plates is indicative of the hydrocarbon degrading potentials of the organism (Hamza *et al.*, 2012).
Culture plates without hydrocarbon introduced into them served as the control.

Modification (Mutation) of isolates

Pure colonies of isolated organisms were modified using a modified method of Zheng and Yujie, (2021). Five plates each of pure colonies of *Bacillus* and *Pseudomonas* species were subjected to X -rays differently for 1second, 2seconds 3seconds, 4seconds and 5seconds respectively. The plates were then wrapped in aluminium foil to prevent photo deactivation. The X-ray treated colonies were then subjected to replica plating by serial dilution on freshly prepared nutrient agar plate. These secondary plates were then incubated at 37^{o}C for 48hours.

Bioremediation of refinery wastewater

A modification of the procedure reported by Chukuka *et al*. (2023) was used for bioremediation of refinery wastewater. Pure strains of parent and modified organisms were grown in nutrient broth at 37^{o}C for 24hours and their microbial count determined. 500ml of 10^{9}cfu each organism was added to 2000ml of

the effluent in a bioreactor while 250ml of each of the isolates were added to 2000ml of the effluent in the bioreactor used for the consortium of organisms. The set up was continuously but slowly aerated. 0.023g of NPK fertilizer was added to the mixture. The temperature, pH and optical density (560nm) were determined after one hour and every three days until the organism attenuated.

Statistical Analysis

Data obtained were statistically analyzed using Pearson" correlation coefficient.

Results

Table 1: Identification of Bacteria Isolates.

TESTS	**A**	**B**	**C**	**D**	**E**	**F**
Cultural Characteristics	Circular rod	Short rod	Round clusters	Coccus	Coccus	Short rod
Pigmentation	Creamy	Greenish	Yellowish	Highly Pinkish	Creamy	Pinkish
Gram reaction	+	-	+	+	+	-
Catalase test	+	+	+	+	-	-
Citrase test	+	+	+	+	+	+
Endospore test	+	-	-	-	-	-
Indole test	-	-	-	+	-	+
H_2S test	-	-	-	-	-	-
Motility test	+	+	-	-	-	+
Oxidase test	-	+	+	-	-	+
Urease test	-	+	+	+	-	-
TSI test	++	-	+++	-	-	+
Organism Identified	*Bacillus sp*	*Pseudomonas sp*	*Micrococcus sp*	*Staphylococcus sp*	*Streptococcus sp*	*Klebsiella sp*

Key: + = Weakly Positive, ++ = Moderately Positive, +++ = Highly Positive, - = Negative

Table 2: Determination of Crude Oil Utilization by *Bacillus sp and Pseudomonas sp*

Concentration (%v/v)	***Bacillus sp***	***Pseudomonas sp***
0.5	++	+
1.0	+	+
1.5	+	+
2.0	+	+
5.0	-	+
Control	-	-

Key: + = Weakly Positive, ++ = Moderately Positive, +++ = Highly Positive, - = Negative

Table 3: Physico-Chemical pararmeters analysis of consortium organisms + fertilizers

	Initial	**Final**	**Amount Degraded**	**% Degradation**
pH	6.89	6.40	0.49	7.1
Total Hydrocarbon Content (mg/L)	0.98	0.20	0.78	79.6

Alkalinity	1.00	0.72	0.28	28.0
Chemical Oxygen Demand (mg/L)	7.63	2.20	5.43	71.2
Biochemical Oxygen Demand (mg/L)	5.81	1.62	4.19	72.1
Turbidity (NTU)	6.40	5.00	1.40	21.9
Total Solid (mg/L)	232	84.0	148	63.8
Total Dissolved Solid (mg/L)	170	60.0	110	64.7
Total Soluble Solid (mg/L)	62.0	24.0	38.0	61.3
Sulphate (mg/L)	53.0	11.2	41.8	78.9
Dissolved Oxygen (mg/L)	1.60	1.80	(0.20)	12.5
Nitrate (mg/L)	0.84	0.42	0.42	50.0
Temperature (mg/L)	20.2	27.8	(7.6)	37.62
Conductivity (mg/L)	113.3	40.0	73.3	64.7
Oil and Grease (mg/L)	1.17	0.64	0.53	45.3

Table 4: Percentage Degradation of TPH in the refinery wastewater (effluent).

	Initial TPH (mg/L)	Final TPH (mg/L)	Amount Degraded (mg/L)	Percentage Degradation
B + E	0.98	0.26	0.72	73.5
B + E + F	0.98	0.18	0.80	81.6
MB + E	0.98	0.25	0.73	74.5
MB + E + F	0.98	0.22	0.76	77.6
P + E	0.98	0.11	0.87	88.8
P + E + F	0.98	0.04	0.94	95.9
MP + E	0.98	0.12	0.86	87.8
MP + E + F	0.98	0.22	0.76	77.6
C + E + F	0.98	0.20	0.78	79.6
Control	0.98	0.98	0.00	00.0

Key: B = *Bacillus,* MB = Mutant *Bacillus,* P = *Pseudomonas,* MP = Mutant *Pseudomonas,* E = Effluent, F = Fertilizer, C = Consortium

The highest degradability of TPH was observed with parent Pseudomonas enhanced with fertilizer (95%) while the least was recorded for B+E (73.5%) as shown in Table 4 above.

Table 5: Reduction in BOD of refinery wastewater (effluent).

	Initial BOD (mg/L)	**Final BOD (mg/L)**	**Amount Reduced (mg/L)**	**Percentage Reduction (%)**
B + E	5.81	2.30	3.51	60.4
B + E + F	5.81	2.24	3.57	61.4
MB + E	5.81	1.72	4.09	70.4
MB + E + F	5.81	1.68	4.13	71.1
P + E	5.81	1.89	3.92	67.5
P + E + F	5.81	1.85	3.96	68.2
MP + E	5.81	1.72	4.09	70.4
MP + E + F	5.81	1.64	4.17	71.8
C + E + F	5.81	1.62	4.19	72.1
Control	5.81	5.81	0.00	0.00

Key: B = *Bacillus,* MB = Mutant *Bacillus,* P = *Pseudomonas,* MP = Mutant *Pseudomonas,* E = Effluent, F = Fertilizer, C = Consortium

The analysis of BOD shows that there was a decrease in BOD values of both parent and mutants from 5.81 to 2.30 and 2.24 for B+E and B+E+F; 1.72 and 1.68 for MB+E and MB+E+F; 1.89 and 1.85 for P+E and P+E+F; 1.72 and 1.64 for MP+E and MP+E+F; 1.62 and 2.77 for A+E+F and control respectively.

Table 6: Statistical Analysis using Pearsons correlation coefficient

	B + E	**B + E + F**	**MB + E**	**MB + E + F**	**P + E**	**P + E + F**	**MP + E**	**MP + E + F**	**C + E + F**	**Control**
B + E	1									
B + E + F	0.99	1								
MB + E	0.60	0.56	1							
MB + E + F	0.63	0.67	0.57	1						
P + E	0.99	0.97	0.63	0.58	1					
P + E + F	0.97	0.97	0.47	0.61	0.97	1				
MP + E	0.63	0.64	0.58	0.92	0.60	0.64	1			

MP + E + F	0.74	0.73	0.74	0.94	0.71	0.70	0.93	1		
C + E + F	0.95	0.97	0.70	0.67	0.94	0.88	0.60	0.73	1	
Control	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	1

Table 7: Values for TVC (log_{10}cfu/ml) during effluent degradation

Samples	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
B + E	6.40	6.60	6.65	6.74	6.68	6.59	6.54	6.58	6.54	6.46	6.73
B + E + F	6.45	6.65	6.66	6.80	6.72	6.59	6.55	6.58	6.56	6.44	6.30
MB + E	6.26	6.61	6.65	6.64	6.62	6.59	6.53	6.56	6.55	6.55	6.06
MB + E + F	6.32	6.69	6.74	6.80	6.76	6.58	6.54	6.60	6.56	6.46	6.23

P + E	6.48	6.56	6.65	6.68	6.70	6.58	6.53	6.56	6.55	6.55	6.06
P + E + F	6.50	6.68	6.74	6.79	6.83	6.59	6.53	6.54	6.56	6.46	6.23
MP + E	6.48	6.55	6.57	6.65	6.54	6.52	6.52	6.48	6.48	6.49	6.11
MP + E + F	6.50	6.56	6.64	6.69	6.61	6.54	6.53	6.49	6.54	6.27	6.30
C + E + F	6.45	6.55	6.65	6.75	6.60	6.61	6.53	6.58	6.56	6.50	6.43
Control	6.39	6.39	6.39	6.39	6.39	6.39	6.39	6.39	6.39	6.39	6.39

Key:

B = *Bacillus,* MB = Mutant *Bacillus,* P = *Pseudomonas,* MP = Mutant *Pseudomonas,*

E = Effluent, F = Fertilizer, C = Consortium

Discussion

The bacteria isolated include *Bacillus sp, Pseudomonas sp., Micrococcus sp., Staphylococcus sp., Streptococcus sp,* and *Klebsiella sp.*
Of all isolates obtained, only *Bacillus* and *Pseudomonas sp* were used in bioremediating the effluent due to their high degree of efficacy in degrading crude oil as reported by Nwachukwu *et al* (2001), who reported that *Bacillus* and *Pseudomonas have high potential in hydrocarbon degradation. Bacillus* and *Pseudomonas* were able to utilize crude oil at different concentrations with *Pseudomanas* having a greater utilization. This difference in degradability could be due to the presence of chemicals in high amount which in turn causes an unfavourable environment for the growth and survival of *Bacillus* as reported by Costa *et.al.,* 2022 and Liu *et. al.,* 2022. Physico-chemical parameters when analysed shows decrease in all parameters except temperature and dissolved oxygen. An increase in the metabolic rate of organisms could result to high temperature while increase in oxygen could be from bioremediation process (Chukuka *et al.,* 2023 and Idise *et al*., 2010b).
It could be deduced that the parent *Pseudomonas* showed higher degradability when enhanced with inorganic nutrients like NPK. The reduction in BOD values was highest in the modified strains. Modified organisms require low energy resulting in high substrate affinity, thus, leading to a reduction in the pollutants concentration and/or recycling of the pollutant to natural compounds in line with the work of Chukuka *et al.,* (2023) and Idise *et al*., (2010b), who reported that inorganic nutrients are vital for the growth and activity of microbial. According to Chukuka *et al*., (2010b), organisms that are modified show better degradability than the parent.
The best performance was obtained with the consortium of organisms. The organisms exhibit a diauxic growth pattern due to the fact that petroleum consists of both linear and aromatic hydrocarbon compounds which usually require different enzymes and biodegradation pathways. Alkanes are usually degraded before the rings in the aromatic chains (Van Hamme *et al.,* 2003).

However, there was an increase in degradability of TPH with the amended substrate than the unamended substrate using modified strains of *Pseudomonas*. While the strains were able to degrade 0.12mg of unmended substrate, the same strain was able to degrade 0.22mg of the amended substrate. This increase in value could result from proliferation of cells due to biostimulation. This also conforms to the work of Idise *et al*., (2010b).

Statistical analysis showed that there was significant difference between the degradability of both parents and mutants, hence the Null hypothesis was rejected.

Conclusion

This study reveals that though refinery effluent is treated, it still contains high microbial load and chemical components which could pose serious health challenge to individuals. However, microorganisms indigenous to refinery effluent are capable of degrading the chemical components of petroleum refinery effluent. The efficacy of degradation increased with the mutants and the addition of chemical fertilizer NPK prior to introduction of the organism.

Recommendation
Effective treatment of refinery effluent prior to discharge into receiving water bodies should be encouraged to avoid pollution and health concerns. Biodegradation of petroleum refinery effluent using mutants strains of microorganisms should be encouraged.

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Conflict of interest
This research work was done without any conflict of interest

References
Ajao, A.T. and Yusuf-Salihu, B. O. (2022).Evaluation of improved bioremediation strategy for the treatment of abattoir wastewater using *Bacillus licheniformis* ZUL012. *Journal of applied science and environmental management.* 26 (6) 1081-1086

Cabral, J. (2010). Water microbiology: Bacterial pathogens and water. *Journal of environmental research and public health.* 7: 3657-3703.

Chukuka, V.I., Onoriasakpobare, F. M., Oyubu, O. L. and Ukolobi, O. (2023). Assessing the capability of X-ray modified strains of *Pseudomonas species* for bioremediation of refinery effluent. *DOU Journal of Scientometrics and Innovations.* 1(1): 88-90.

Costa, C., Santos, A. and Vega, M. A.(2022). Kinetics of Arab Light Crude Oil Degradation by Pseudomonas and Bacillus Strains. *Water.* 14 (23) 3802.

Emoyan, O. O., Peretiemo-Clark, B. O., Tesi, G. O., Adjerese, W. and Ohwo, E. (2021). Occurrence, origin and risk assessment of heavy metals measured petroleum tank-farm impacted soil. Soil and sediment contamination: *An international journal.* 30(4) 384-408.

Govind K., Sai P., Archna S and Ghanshyam P. (2019).Bioremediation of petroleum hydrocarbon-polluted soil using microbial enzymes. *Science Direct- Elsevier*. 307-319.

Hamza, U.D., Mohammed I. A and Abdullahi, S. (2012). Potentials of bacteria isolates in bioremediation of petroleum refinery wastewater. *Journal of applied phytotechnology in environmental sanitation*. 1(3) 131-138.

Hamza, R., Nadia, A., Sadia, R., Arooj, A., Maham, I., Asim, H., Muhammad, B. and Hafiz, M. N. I. (2023). Genetically engineered microorganisms for environmental remediation. *Chemosphere-elsevier*. 310:136751

Idise, O. E., Ameh, J. B., Yakubu, S. E., Okuofu, C. A. and Ado, S. A. (2010b). Determination of the genetic marker of the mutagenized strains of *Pseudomonas aeruginosa* and *Bacillus cereus*isolated from effluent of petroleum refinery. Bayero Journal of Pure and Applied sciences. 3 (1) 180-182.

Jewo, A. Oyubu, L. O., Anomoharan, E. E. and Animam, B. (2023). Bacteriological and physicochemical assessment of treated petroleum effluent obtained from some petrochemical industries in Warri, Nigeria using activated rice husk. *American journal of environmental protection.* 11(1) 15-24.
Koshlaf, E. and Bail, A. S. (2017). Soil bioremediation approaches for petroleum hydrocarbon polluted environments. *AIMS Microbiology*. 3(1) 25-49.

Liu, S., Sun, R., Cai, M., Kong, Y., Gao,Y., Zhang, T., Xiao, X., Qjao, Y., Xue, J. and Huang, G. (2022). Petroleum spill bioremediation by an indigenous constructed bacterial consortium in marine environments. *Ecotoxicology and Environmental Safety* 241:113769.

Mace, G. B., Deborah, N. V., Ron A. H. and Un, H. Y. (2020). Long-Term Ecological Impacts from Oil Spills: Comparison of Exxon Valdez, Hebei Spirit, and Deepwater Horizon. *Environmental Science and Technology54* (11) 6456-6467.

Maheen, K., Hayat, U., Aasma, G., Tehmina, S., Zarif, G., Munzer, U., Maliha, S., Muhammad, W. A., Nida, N. K., Tayyaba, B., Saman, M. and Ayesha N. (2022). Biodegradation of petroleum hydrocarbons and the factors affecting rate of biodegradation. *American journal of Biomedical Science and Research.*16(1) 2182.

Ojo, T. A., Uwanta, L. I., Ezeokoli, C. M., Okoye, P. A and Ezemba, C. C. (2021).Biodegradation of crude oil by hydrocarbon degrading bacteria isolated from contaminated soil samples in selected tank farm areas in Apata, Ibadan. *Journal of global ecology and environment*. 13 (4) 102-109

Zheng, W. and Yujie, Z. (2021). Synonymous point mutation of *gtfB* gene caused by therapeutic x-ray exposure reduced the biofilm formation and cariogenic abilities of *Streptococcus mutans.* Cell and Bioscience 11:91