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Biodegradation of Total Petroleum Hydrocarbon by Aerobic Heterotrophic Bacteria Isolated from Crude Oil Contaminated Brackish Waters of Bodo Creek

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Abstract

Bodo creek is located in Gokana Local Government Area of Rivers state and is characterised by brackish water which is heavily contaminated with crude oil. Water samples collected from the creek were taken to Environmental Microbiology Laboratory of University of Portharcourt for isolation of aerobic heterotrophic bacteria using serial dilution spread plate technique. Isolation of morphologically distinct pure cultures was done and the isolates obtained were identified molecularly on the basis of 16S rRNA gene sequence analysis. 16S rRNA sequencing for identification of the isolates generated sequences ranging from 500 bp to 1700 bp and a 250 bp size PCR amplified fragment. The nucleotide sequence of the 16S rRNA gene was compared with published 16S rRNA sequences using BLAST search at the data base of National Centre for Biotechnology Information (NCBI). Biodegradation of petroleum hydrocarbon test on the isolates was conducted using Bony light crude oil obtained from Shell Petroleum Development Company and monitored using GC-FID (Agilent 6890 model) for 49 days. The initial quantity of TPH was 24091 and 6706 mg/l on day 0 for aerobic heterotrophic treatment and crude oil contaminated control and decreased progressively to 212.8 and 1174 mg/l respectively on day 49 indicating biodegradation in the treatment and control. Loss of TPH was statistically significant using one way analysis of variance (ANOVA) $p < 0.05$ with time. Model for predicting TPH loss was developed and rate of biodegradation of the isolates determined using Trend line method of Microsoft Excel, 2010. The growth of bacteria cells increased progressively with decrease in TPH implying that the bacteria were responsible for biodegradation. Further application of bioremediation strategies in Bodo creek for biostimulation of the crude oil biodegrading bacteria for reclamation and restoration of an efficient ecosystem structure and function is suggested.

Keywords: Biodegradation; Aerobic heterotrophic bacteria; Total petroleum hydrocarbon; Brackish water; Molecular analysis

Introduction

The contamination of surface water with petroleum hydrocarbons is among the most severe environmental problems facing Nigeria [1,2]. Hydrocarbons can enter water through direct spills or from a spill originally occurring on land and subsequently reaching water bodies through the effects of wind, rain, surface or sub-surface flow thus causing physical and chemical effects in water [2]. The presence of mere traces of a toxic hydrocarbon such as benzene may render water unfit for human consumption [3,4]. Hence, there is the need to remediate their impact as soon as they enter the water body.

Biodegradation of petroleum hydrocarbon in the environment is a complex process. The quantitative and qualitative aspects depend on the nature and amount of the oil or hydrocarbon present, the composition of autochthonous microbial community [5,6]. Hydrocarbon degrading bacteria are widely distributed in marine freshwater, soil habitat and their use in bioremediation of hydrocarbon contaminated soils, which exploits their ability to degrade and/or detoxify organic contaminants, has proven to be an efficient, economical, versatile and environmentally friendly treatment [7].

There is a broad spectrum of distribution of bacteria that have ability to degrade or transform hydrocarbons [8]. [5,9,10] isolated varying types of bacteria from brackish water capable of petroleum hydrocarbon degradation and these include members of the genera *Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Brevibacterium, Corynebacterium, Enterobacter, Escherichia, Flavobacterium, Norcadia, Pseudomonas, Staphylococcus,* and *Vibrio.*

Brackish waters have a higher percentage of salinity over freshwater and the salt concentrations are reported to constitute a stressful agent for most of the known living organisms. Organisms that grow best in such environment are called halophiles, while the ones that grow in non-saline environments but are able to grow in hypersaline environments are regarded as halotolerant organisms. Bodo creek of Bodo community in Ogoniland of Gokana LGA of Rivers State is characterised by brackish water and has been contaminated with petroleum hydrocarbons due to oil exploration activities and spillages. Gokana LGA is over the major oil producing areas in Rivers state and is located within the south east senatorial district of the state, comprising both the riverine and upland communities [2]. The aim of our study was to isolate and identify aerobic heterotrophic bacteria from brackish waters of hydrocarbon polluted Bodo creek and test them for biodegradation potentials, which justifies the relevance of the study.

Materials and Methods

Sampling size, sampling technique and isolation procedures

Ten samples of surface water and sediment samples were collected at distance of 50m apart from the designated points and 20m from the

coast. The water samples were collected on the surface and transported to the environmental microbiology laboratory of the University of Port Harcourt for isolation. The bacteria were isolated using serial dilution as described by Cheesebrough [11] with sterile water and the 10^{-3} dilution was considered and the duplicate of the isolates plated on a composed artificial sea water medium containing 5.6 g/l $Mgcl_2.6H_2O$, 6.8 g/l $MgSO_4.7H_2O$, 1.47 g/l $CaCl_2.2H_2O$, 0.66 g/l KCl and 0.09 g/l KBr and autoclaved after which 0.15 and 0.2 g/l of KH_2PO_4 and NH_4Cl solutions were added. Autoclaved 1 ml/l solutions of trace elements selenite, tungstate and vitamins were then added and agar was then added to prepare a solid medium and the salinity adjusted to reflect the saline condition of the samples [12]. Pure cultures of the resulting morphologically distinct colonies were isolated from the first culture and plated on solid medium and incubated for 24 hours for molecular identification.

Molecular characterization of bacteria isolates

DNA extraction of the isolates was done using the CTAB method, as described by Moore and Dowhan [13]. DNA electrophoresis, using agarose gel electrophoresis to determine the quality and integrity of the DNA by size fractionation, PCR analysis using 16S universal primers for bacteria [14] and DGGE analysis of PCR chain reaction amplified genes coding for 16S rRNA. The 16S rRNA sequences of the strain were analysed using ARB software [15] and the sequences of the isolates generated were submitted to NCBI Genbank for assigning of accession numbers.

Biodegradation experiment with bacteria isolates

Aerobic heterotrophic bacteria aliquot was prepared by transferring a loopful of 24 hr culture of each isolate into 400 ml of sterile nutrient broth in 500 ml Erlenmeyer flask and incubated for 24 hrs. Treatment of brackish water sample with AHB was prepared by aseptically transferring 200 ml of aerobic heterotrophic bacteria aliquot into 500 ml of sterile distilled water in to a separate 1000 ml flask and was standardized using 0.5 M macfarland solution. The water container for the experimental set up was filled with 11 litres of brackish water not contaminated with crude oil. This was subsequently contaminated with 32300 ppm of sterile Bony light crude oil sample obtained from shell petroleum development company and inoculated with aerobic heterotrophic bacteria while the control was contaminated with same volume of crude oil but left untreated with aerobic heterotrophic bacteria. AHB treated set up was further treated with $CuSO_4$ and 0.25 mg/ml of nystatin to prevent cyanobacteria and fungal activities in the setup whereas the control was left untreated. The containers for the treatments were washed with detergents and rinsed severally with distilled water prior to the experimental set up.

Biodegradation activities were monitored for a period of 49 days for petroleum hydrocarbon degradation using Gas chromatography.

Results

Total petroleum hydrocarbon (TPH) biodegradation experiment

In an attempt to determine the capability of isolated aerobic heterotrophic bacteria from Bodo creek in biodegradation of total petroleum hydrocarbon, water samples from a section of the uncontaminated Bodo Creek adjudged to be unpolluted by crude oil as observed in our preliminary result of the Gas chromatography

conducted on it and physical observation were contaminated with known volume of Bony light crude oil. The TPH present were C-9, C-10, C-11, C-12, C-13, C-14, C-15, C-16, C-17, C-18, C-19, C-20, C-21, C-22, C-23, C-24, C-26, C-30 and C-32 for the treatment on day 0. The total amount of TPH after contamination of water with crude oil showed that the treatment had TPH of 24091 mg/L and Control 6706 mg/L for the first day of contamination. See the chromatograms in Figures 1-4 for quantity of TPH for the first and last day of sampling respectively. TPH for AHB reduced significantly to 212.8 mg/L and control 1174 mg/L. It was observed that the consortium of AHB degraded TPH throughout the period of the experiment whereas no degradation was observed for the control on day 42 and 49 though bacteria growth peaked throughout the period of the experiment for the treatment and the control (Figures 5 and 6). Figure 7 depicts modelling of TPH degradation by the treatment options using the Trend line Method of Microsoft Excel. 2010. It was observed that aerobic heterotrophic bacteria consortium was able to degrade TPH exponentially. The curves indicate the presence of volatile compounds in the contaminated samples because of the observed initial sharp decrease in TPH in the treatment. The coefficient of determination (R^2) is an indication of how well the model can be used to predict TPH degradation with time for the treatment options. AHB coefficient of determination (R^2) for the quantity of TPH left per time using the equation TPH (Y) = $19799e^{-0079x}$ where x is the time. The coefficient of determination of values (R^2) (goodness of fit = 0.921) which implies that if the experiment is repeated, there is possibility of obtaining the same result at 92.15% confidence level for AHB and can be used to determine the quantity of TPH left. This same pattern applied to control 96.51% (0.963) with a different equation; TPH, y= $20156e^{-0.06x}$ for control, where x= time (Figure 8). The rate of TPH degradation was monitored for all the treatments in the experiment. The rate of degradation was high at the beginning of the experiment but decreased with time due to substrate exhaustion resulting from substrate utilization or degradation by microorganisms involved. A negative relationship was observed between the rate of TPH degradation and time (days), r value of -0.91. Figure 9 shows the daily rate of TPH degradation by AHB per each day, using the equation Y (rate of TPH degradation) = $27900 x^{-1.514}$ where x = time. No pattern of TPH degradation was observed for the control as shown in Figure 10.

16S rRNA sequencing for identification of the isolates generated sequences ranging from 500 bp to 1700 bp and a 250 bp size PCR amplified fragment. The nucleotide sequence of the 16S rRNA gene was compared with published 16S rRNA sequences using BLAST search at the data base of National Centre for Biotechnology Information (NCBI).
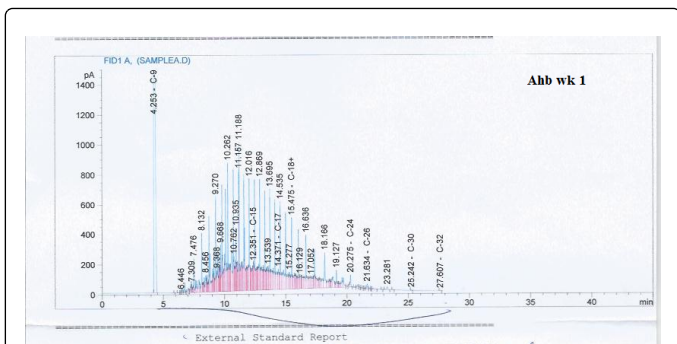
Figure 1: Chromatogram of crude oil contaminated water treated with aerobic heterotrophic bacteria on day 0
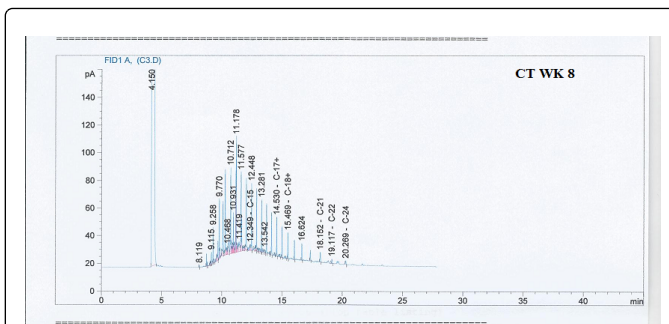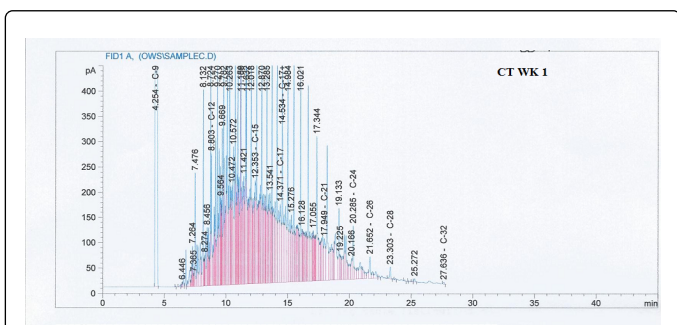

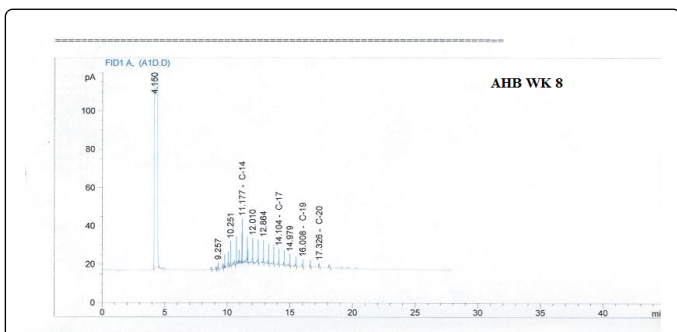Figure 2: Chromatogram of crude oil contaminated control (water) untreated with any study microorganism on day 0

Figure 3: Chromatogram of crude oil contaminated water treated with aerobic heterotrophic bacteria on day 49

Figure 4: Chromatogram of crude oil contaminated control (water) untreated with any of the study microoganism on day 49
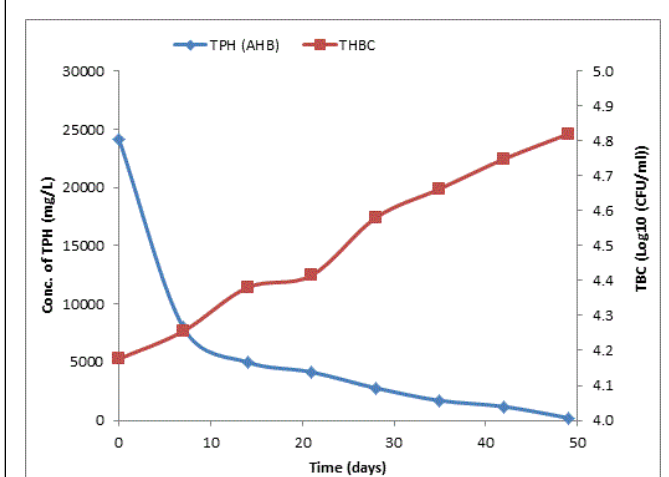

Figure 5: TPH degradation by aerobic heterotrophic bacteria
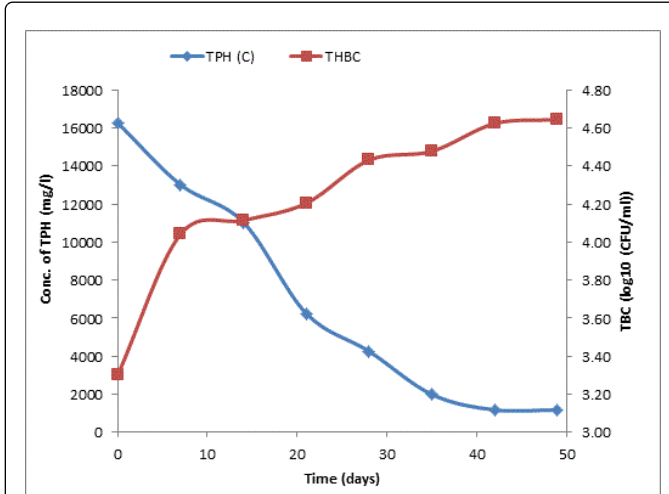

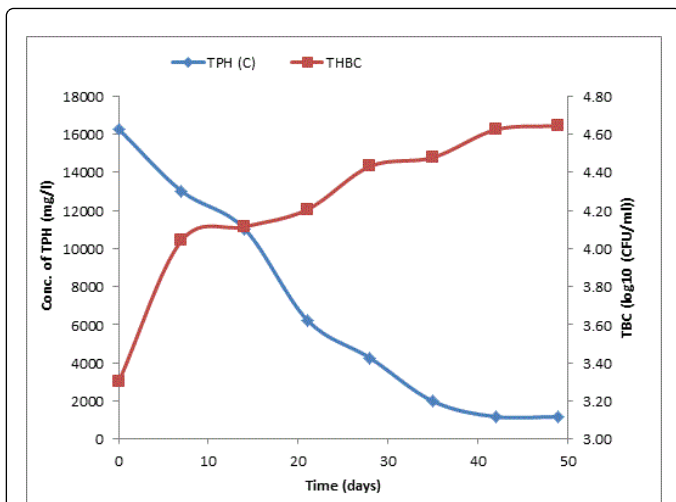Figure 6: TPH degradation by untreated crude oil contaminated control

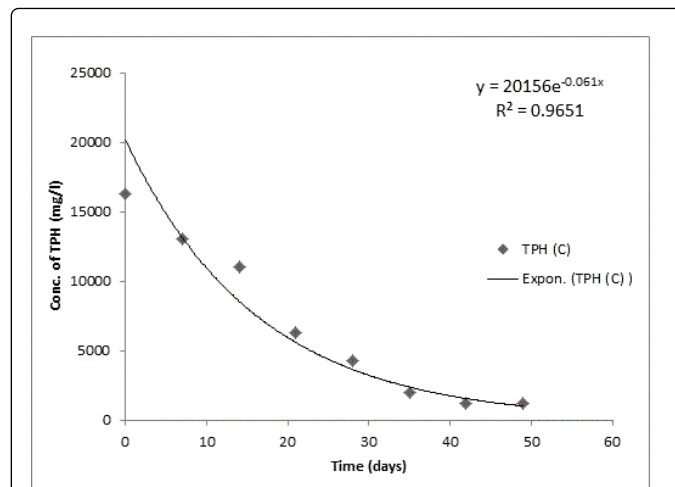Figure 7: Model for TPH degradation by AHB

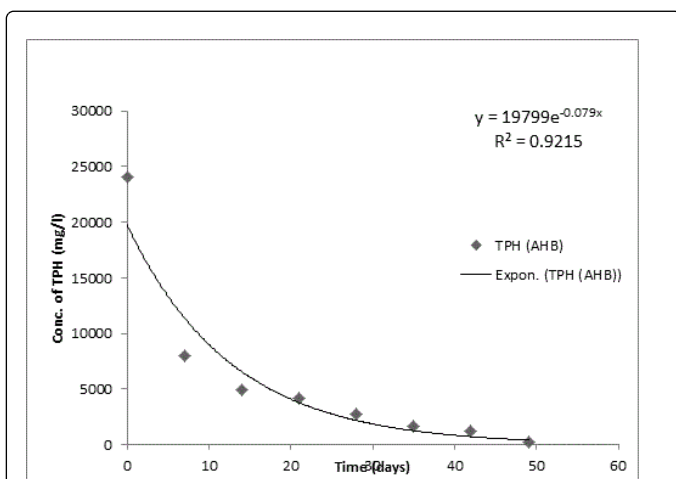Figure 9: Rate of TPH biodegradation by AHB

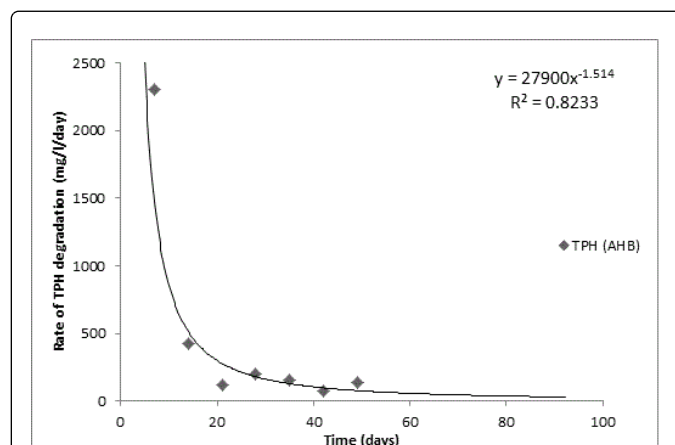Figure 8: Model for TPH degradation by Crude oil Contaminated Control

Figure 10: Rate of TPH biodegradation from crude oil contaminated control

Discussion

Biodegradation of petroleum hydrocarbons in saline aquatic environment by aerobic heterotrophic bacteria has been reported by other researchers. Abed and Koster [16] examined degradation of hydrocarbons by aerobic heterotrophic bacteria in a cyanobacteria mat and found out that they were chiefly responsible. Similar studies were carried out and the same observations have been reported by other researchers in aquatic environment [5,17-24].

The result of the study clearly demonstrated the ability of the consortium of the isolated aerobic heterotrophic bacteria to degrade total petroleum hydrocarbon which justifies the essence of their presence in this crude oil contaminated brackish water habitat. Previous studies have reported on biodegradation of petroleum hydrocarbons in both terrestrial and marine environment under oxic conditions [5,24-26].

The bacteria isolated and identified with their accession numbers include; *Alcaligenes faecalis* (KF056900.1), *Bacillus pumilus* (KF717600.1), *Bacillus pumilus* (JX680132.1), *Bacillus pumilus* (HQ334985.1), *Myroides odoratimimus* (GU186112.2) *Bacillus cohnii* (KC813164.1), *Citrobacter murliniae* (KF254752.1), *Bacillus pumilus* (HM744710.1), *Bordetella sp* (KF601910.1), *Lysinibacillus fusiformis* (KC329831.1), *Bacillus pumilus* KF387715.1), *Myroides pelagicus* (NR041042), *Bacillus pumilus* (JQ833612.1) and *Enterobacter cloacae* (JQ832514.1) and belonged to the families of *Enterobacteriaceae, Bacilliceae, Alcaligenaceae, Pseudomonadaceae, Flavobactericeae and Planococcaceae* which members have been implicated in petroleum hydrocarbon biodegradation even at higher salinities compared to the to the present area under study [27]. Microbial degradation is the major and ultimate natural mechanism by which petroleum hydrocarbon pollutants can be cleaned from the environment [17,28,29]. It is proven by researchers to be more effectively done by a consortium of bacteria which out performs single species isolated even

from the consortium and can occur under oxic and non oxic conditions [30].

Total petroleum hydrocarbon degradation as observed in this study provides evidence that the consortium of bacteria isolated from the crude oil contaminated and the uncontaminated site used as control both possess an inherent capability for biodegradation of petroleum as monitored using GC-FID. This corroborates report of on the isolation of hydrocarbon degraders from environment [30-32]. The reduction in TPH was more fiercely observed for the treatment and control during the first seven days as observed from Figure 5 and 6 implying that volatile components of the crude oil had evaporated. TPH loss was statistically significant ($p < 0.05$) with time for the treatment and control throughout the experimental period using one way analysis of variance though samples on day 42 and 49 showed no decrease in TPH.

The growth of bacteria in the treatment and control increased progressively with decrease in TPH throughout the 49 days period monitored. The continued bacteria growth in the control irrespective of stalled biodegradation of TPH on day 42 and 49 could be attributed to other favorable environmental factors, presence of limiting nutrients which were adjusted to mimic the natural environmental conditions from which the bacteria were isolated and other metabolites suspected to have been generated during the biodegradation which our subsequent research seeks to unravel. A negative correlation between growth of aerobic heterotrophic bacteria and TPH loss was observed for both the treatment and the control ($p < 0.05$) implying that bacterial growth increased with decreasing quantity of TPH for the treatment and the control. Latha and Kalaivani [33] reported a correlation between increased oil degradation to an increase in cell number of bacterial indicating that the isolates were responsible for the degradation which corroborates the result of our finding.

The rate of biodegradation has been widely reported to be affected by a number of factors. Our study considered these factors and adjusted our set up to reflect the factors at the site where the pollution occurred and samples were obtained in order to ascertain near accuracy the activities of the autochthonous bacteria at the pollution site. The result established the fact that bacteria isolated from crude oil polluted Bodo creek have efficient ability for utilizing crude oil as a source of carbon and energy. Application of bioremediation strategies such as biostimulation and bioaugmentation as reported in various laboratory researches should be applied to field experiment to breach the gap between bench findings and real solutions to environmental challenges that stimulated the research initially for reclamation of such environment for efficient ecosystem balance and function.

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