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Bacteria Deterioration of Polyaromatic Hydrocarbon (PAH) Component of Premium Motor Spirit (PMS) from Underground Storage Tanks

[1]Sanyaolu, Adeniyi A.A, [2]Kolawole-Joseph, O.S. and [3]Akinjayeju, Doyinsola O.
[1]Departmen of Botany and Ecological Studies, Faculty of Science, University of Uyo,
[2]Microbiology Unit, Department of Biological sciences, School of Science, Yaba College of Technology, Akoka Lagos, Nigeria
[3]Department of Microbiology, Federal University of |Technology, Akure, Nigeria

**Corresponding Author: adeniyisanyaolu@uniuyo.edu.ng, (+23480-5309-1616)*

Abstract

The study of Biodeterioration of Premium Motor Spirit (PMS) by bacterial deteriogens was conducted to ascertain the level of spoilage on petroleum samples obtained from two Petroleum Service Stations in Lagos State, Nigeria. The Spread plate technique was employed for the enumeration of the microorganisms. The samples were cultured on petri-dishes containing nutrient media and were incubated at a temperature of 30°C for 48 hours for bacterial growth. The bacterial isolates were further tested for hydrocarbon utilization potential with a modified mineral salt solution at room temperature for 48 hours. The bacterial isolates were aseptically reintroduced into freshly sterilized PMS samples and their deteriorating actions on the Polyaromatic hydrocarbon (PAH) component of these samples was verified using the gas chromatographic technique. PMS samples were sterilized using the membrane filtration technique. Results for the isolation showed a high range of total viable counts of Bacterial species such as *Pseudomonas aeruginosa, Clostridium sporogenes, Bacillus subtilis, Aerococcus viridians, Staphylococcus aureus and Mycobacteria* spp. associated with the PMS samples from the underground storage tanks. *Bacillus subtilis* was capable of causing a remarkable reduction in the PAH components (naphthylene, acenaphthalene and fluorine) of the PMS samples. The abundance in microbial growth in the petrol substrates was probably as a result of heavy contamination of the samples when in the underground storage tanks, and also the presence of moisture. It is therefore recommended that adequate spoilage-control measures, such as the prevention of the infiltration of moisture should be taken in order to prevent bio-attack of the petroleum products.

Keywords: **Biodeterioration, Bacterial isolates, Premium motor spirit, Polyaromatic hydrocarbon, Bio-attack.**

Introduction

Biodeterioration of petroleum products is the process where complex microbial communities, consisting hydrocarbon oxidizing microorganisms use the hydrocarbons that are present in the PMS as a source of food and ultimately reducing them (Obahiagbon *et al*, 2014). Biodeterioration of crude oil and oil fuels is recognized as a serious economic and environmental problem the world over. It is not possible to completely exclude the invasion of microorganisms in oil and fuels, either during or after drilling or in storage tanks (Speight, 2015). Complex microbial communities consisting of both hydrocarbon oxidizing microorganisms and bacteria using the metabolites form an ecological niche in these Oil and Gas installations (Zhou *et al*., 2011). As in biodegradation, bacteria and fungi are also the most implicated microorganisms in the process of biodeterioration (Yemashova *et al*., 2007). These microorganisms enter into petroleum products such as petrol and diesel through the air route and microorganisms such as bacteria and spores of fungi are often present in the air (Atlas, 1991). The problem of biodeterioration or biofouling of crude oil and its derivatives has been a subject of immense interest to scientists and engineers for many decades (Hill, 1967; Odier, 1976; Neidle *et al*., 1989; Samanta *et al*., 1999; Yemashova *et al*., 2007 and Sanyaolu *et al*., 2012) because of its huge ecologic and economic implications (Roling *et al*., 2003; Stanley *et al*., 2016).

There are some implications of biodeterioration in petroleum products, which include; deteriorating microorganisms secreting of some weak molecular weight substance (e.g. biosurfactants) that react with petroleum and accelerate the formation of particles which makes fuel more corrosive (Bento and Gaylarde, 2001). Microbial deterioration of aviation fuel has been reported to have caused crashes and explosions in aircrafts and this happens because of the secretions by the microorganisms which make the fuel corrosive (Jida *et al*., 1998). Also, the presence of bacteria in diesel engines has been reported to be one of the leading causes of engine breakdown (Gaylarde *et al*., 1999). Furthermore microbial deterioration causes destabilization of the chemical nature and structure of the petroleum hydrocarbon making it almost impossible to get other products from the petroleum hydrocarbon (Bento and Gaylarde, 2001). Deteriorating microorganisms destroy the quality and integrity of petroleum and its products. Deteriorating microorganisms in oil fuels also produce biosurfactants, biosulfates, detergent molecules and hydrogen sulphide molecules which compromise the quality and efficiency of the fuel (Maerki *et al.*, 2006). They also produce slime, thereby precipitating fouling, a severe filter plugging, blocking of fuel lines, nozzles of injectors which culminates in excessive wear and failure in engines and system component. Other important concerns associated with the microbial

deterioration of fuels in engine systems are increased water partitioning into the fuel-phase, increased fuel and bottom-water corrosives, and fuel souring (Maerki *et al.*, 2006). It is on the above premises that this research work was conceived, thus the aim of this work is to evaluate the extent of deterioration of petrol (PMS) by some bacterial species, through the isolation and identify species of bacteria found in samples of PMS in some parts of Lagos metropolis, verify the possibility of the bacteria isolates growing in the PMS samples and to determine the possibility and extent of deterioration of some of the PAH component of PMS by one of the bacterial isolates.

Materials and Methods

Samples Collection

Premium motor spirit (PMS) samples were collected from two filling stations at two locations remotely situated from each other, namely MRS Service Station, Badagry roundabout at Badagry, Lagos State, and Oando Filling Station, Fadeyi, Shomolu Local Government Areas of Lagos State, Nigeria, with geographical coordinates (+32ft N.06 25.842 E002 53.738) and (+41ft N.06 31.453 E003 22.069) respectively. The samples were collected into pre-sterilized containers. After collection, the samples were taken to the laboratory and cultured within the first 3 hours (after collection) to determine the bacterial species that were present in the samples.

Sample Preparation for Bacterial Counting

Seven grams of nutrient agar was measured out and dissolved in 250 ml of distilled water. This was then autoclaved for 15 minutes. The prepared nutrient agar was thereafter aseptically poured into ten sterile petri-dishes (Five each for the PMS samples from each location). The plates were then sealed using masking tape, after which the samples were then incubated for 72 hours. After 72 hours of incubation, Gram staining was carried out on all the plates that showed visible growth. Sterile grease free glass slides were cleaned with cotton wool soaked in ethanol. A spirit lamp was switched on and used to sterilize the work environment and the inoculating loop. Then, a loop full of distilled water was placed on the slide and the loop was flamed. After which loop full of each organism were picked and emulsified with the distilled water. It was then air-dried and heat fixed by passing the slide over the flame for 3-5 times. The Gram's staining techniques followed standard method after which the slides were counter stained with carbol fuschin for 2 minutes, after which they were air-dried and viewed under the microscope using the immersion oil objective.

Sterilization of Petroleum Product Samples

The membrane filtration technique as described by Sanyaolu *et al.* (2012) was used. The Membrane filter (Millipore 47mm x 1104700) used was first sterilized by wrapping it in aluminum foil and placing it in an autoclave at 121°C for 15 minutes. It was thereafter allowed to cool and the PMS was poured (at different times after each round of sterilization) from the top lid, and allowed to drain through the filter into a pre-sterilized collecting container attached to the membrane filter. The entire exercise was done under an aseptic condition in a UV room. Prior to the sterilization however, isolation of the bacterial species from the

PMS was performed. This process involving the use of the membrane filter only allows the filtration of bacteria and other organisms larger than the pore size, thus rendering the samples sterile.

Hydrocarbon Utilization Ability of the Bacterial Samples

A modified method of the enrichment procedure in a Minimal Salt Solution (MSS) as described by Norma *et al.*, (2008) was used in the estimation of the bacterial species that are capable of utilizing the petroleum hydrocarbon samples. The MSS was prepared thus: 2.13g of Na_2HPO_4 (disodium hydrophosphate), 0.5g of NH_4cl (Ammonium chloride), 1.3g of KH_2PO_4 (Potassium hydrogenphosphate), 0.2g of $MgSo_4.7H_20$ (Magnesium sulphate heptahydrate) and 0.55g of yeast were all dissolved in 1000 ml of sterile distilled water. The pH was adjusted to 6.5 (normal pH for bacteria growth). Using a sterile pipette, 10 ml of the MSS solution was dispensed into each of the twenty four test tubes containing 2 ml each of the pre sterilized PMS. Using a 2mm cork borer, the previously isolated bacteria samples were picked and dropped under aseptic conditions into the sterile MSS and PMS mixture. These test tubes were plugged with cotton wool wrapped in aluminum foil so as to facilitate a maximum aeration of the bacteria cell/MSS and PMS mixture as well as to prevent a cross contamination of the experimental set up.

The eight test tubes were set up using a test tube rack as follows:

- ✓ 10 ml Mss + 2 ml PMS (Shomolu sample) + *Pseudomonas aeruginosa*
- ✓ 10 ml Mss + 2 ml PMS (") + *Bacillus subtilis*
- ✓ 10 ml Mss + 2 ml PMS (") + *Acetobacter* sp
- ✓ 10 ml Mss + 2 ml PMS (Badagry sample) + *P. aeruginosa*
- ✓ 10 ml Mss + 2 ml PMS (") + *B.subtilis*
- ✓ 10 ml Mss + 2 ml PMS (") + *Acetobacter* sp
- ✓ Control samples A (from each of the 2 locations) containing 10 ml of MSS + 2 ml of sterile PMS – bacteria cells.

Each of these treatments was replicated 3 times to give a total of 24 test tubes in all.

Determination of the Growth of Bacterial in PMS

This was done by measuring the optical density of the different Treatments by determining the absorbance. Absorbance of the samples was measured using the Spectrophotometer (model T80 UV/VIS by PG Instruments Limited) at wavelength of 520 nm. The growth rate of the bacteria cells in the MSS and PMS media was measured as a function of the turbidity of the media every 3 days for a total of 21 days using this spectrophotometer. Day 0 (zero) data were taken in all cases less than 24 hours (between 18-22 hours) after inoculating the Treatment samples with the bacterial cell.

Gas Chromatographic Analysis

Exactly 5ml of the PMS sample was transferred into a 50 ml separating funnel, and 5ml of redistilled dichloromethane was added. The separating funnel was shaken vigorously for about 2 minutes with periodic venting to release vapour pressure. The organic layer was allowed to separate for 10 minutes and was recovered into the 50 ml tank. The aqueous layer was

re-extracted twice with 2 ml of the extractant - redistilled dichloromethane. The combined extract was dried by passing through the funnel containing the anhydrous sodium sulphate. The dried extract was concentrated with a stream of nitrogen gas.

The concentrated extract was then separated into the aliphatic profiles and poly aromatic hydrocarbon profiles by packing the glass column with activated alumina, neutral and activity grade1. The treated alumina 10 ml was packed into the column and cleaned properly with redistilled hexane. The extract was poured onto the alumina and was allowed to run down with the aid of the redistilled hexane to remove the aliphatic profiles into a pre-cleaned 20 ml capacity glass container. The aromatic fraction was recovered by allowing the mixture of hexane and dichloromethane in the ratio 3:1 and finally removed the most polar PAH by removing with the dichloromethane into the pre-cleaned boro-silicate beaker. The mixture was concentrated to 1.0 ml by stream of the nitrogen gas before the gas chromatography analysis.

Statistical Analysis of Data

The values reported were the means values from the three replicates. These mean values were analyzed statistically using the two paired T-test using the IBM SPSS 20.0 statistical software. Here, the mean values obtained for each of the respective controls were compared with the Treatments.

Results and Discussion

Results

The result of the isolation of bacteria from the samples revealed a total of six bacteria species in the PMS, namely: *Pseudomonas aeruginosa, Clostridium sporogenes, Bacillus subtilis, Aerococcus viridians, Staphylococcus aureus and Mycobacteria* sp

The results of the optical activity of the bacteria cells in the PMS samples (which depicts the rate of the growth of these organisms in these samples) are presented in Table 1. The result shows that there was no significant (P = 0.05) difference in the rate of growth among all the isolates from both locations and the control samples for Day 0. However, by Day 21, there were significant (P = 0.05) differences in the growth of each isolate relative to the growth at Day 0. In the same vein, at Day 21, the growth of each isolate was significantly (P = 0.05) higher than the corresponding control sample. Moreso, the results as shown in Table 1 revealed that there were no significant (P = 0.05) differences amongst the Treatment samples at Day 21.

Table 1: Optical Density (520 nm) of Bacterial contaminated PMS samples at Days 0 and 21

Bacteria species	Optical density of PMS at Day 0	Optical density of PMS at Day 21
A. viridans (Shomolu isolate)	1.6133[a]	1.6266[b]
B. subtilis (Shomolu isolate)	1.6033[a]	1.6200[b]
P. aeroginosa (Shomolu isolate)	1.6066[a]	1.6233[b]
Control (Shomolu sample)	1.6066[a]	1.6033[a]
A. viridans (Badagry isolate)	1.6056[a]	1.6156[b]
B. subtilis (Badagry isolate)	1.6060[a]	1.6166[b]
P. aeroginosa (Badagry isolate)	1.6066[a]	1.6200[b]
Control (Badagry sample)	1.6056[a]	1.6033[a]

Mean values carrying the same superscript along the same column are not significantly different at P=0.05 while values carrying different superscripts along the same row are significantly different at P=0.05.

The results from Figures 1-3 show the PAH analysis of the PMS, the components that were detected in the PMS samples were acenaphtylene, naphthalene and fluorine. The results from Figure 1 shows that by day 21, the concentration of acenaphtylene in the PMS sample had been reduced by *B. subtilis* to 0.172 Mg/l from 0.578 Mg/l present at Day 0. The results in Figure 2 shows that *B. subtilis* had equally achieved a reduction in the concentration of naphthalene from 2.545 Mg/l at Day 0 to 0.63 Mg/l at Day 21. While the results from Figure 3 shows that the fluorine concentration in the PMS samples had been reduced to non detectable limits by *B. subtilis* in the Day 0 and Day 21 samples as opposed to the sterile (Control) samples which had a fluorine concentration of about 0.00012 Mg/l. In all the cases in Figures 1-3 however, the concentration of each of the PAH component of the PMS in the Control samples were higher than what was observed and obtained in all the *B. subtilis* contaminated TPMS Treatment samples

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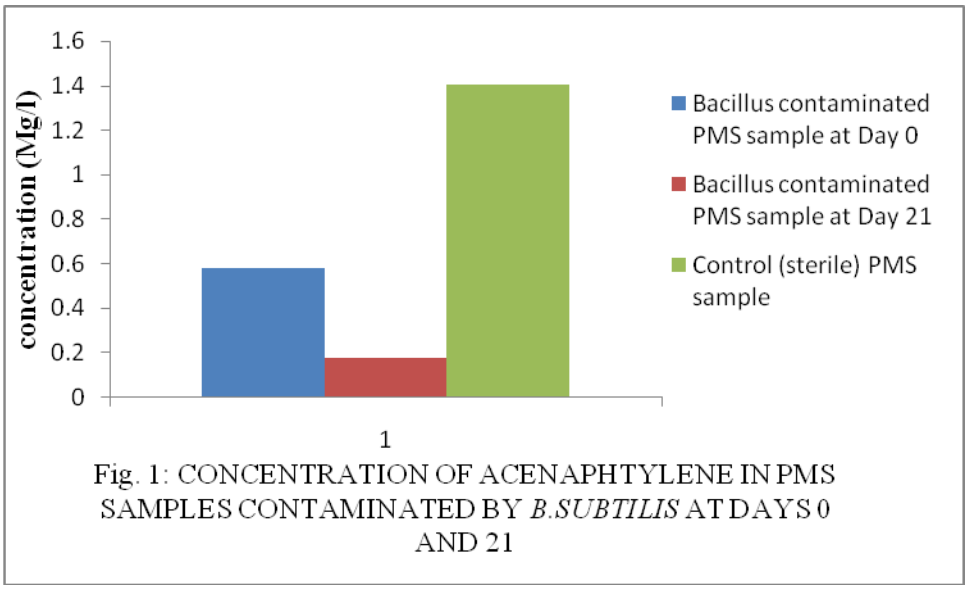

Fig. 1: CONCENTRATION OF ACENAPHTYLENE IN PMS SAMPLES CONTAMINATED BY *B.SUBTILIS* AT DAYS 0 AND 21

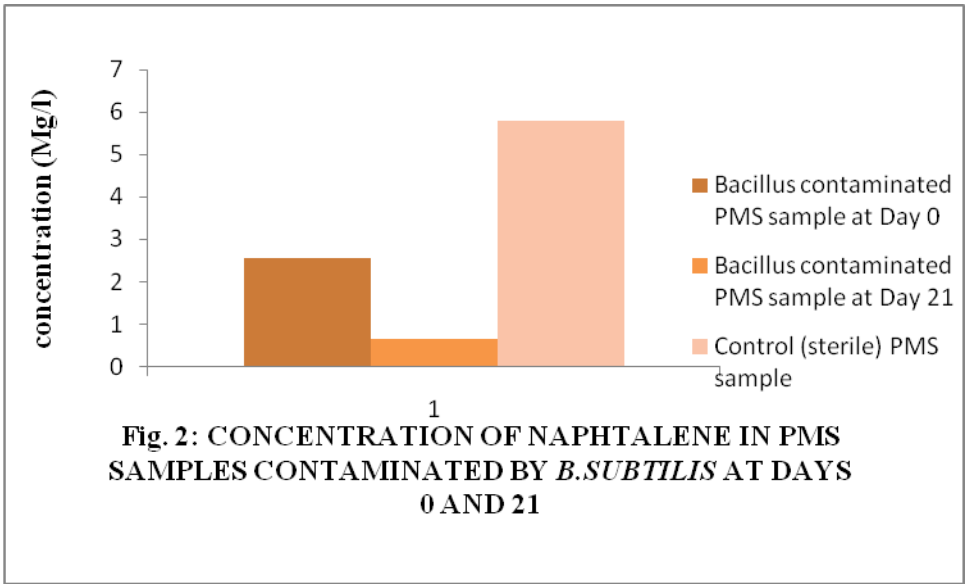

Fig. 2: CONCENTRATION OF NAPHTALENE IN PMS SAMPLES CONTAMINATED BY *B.SUBTILIS* AT DAYS 0 AND 21

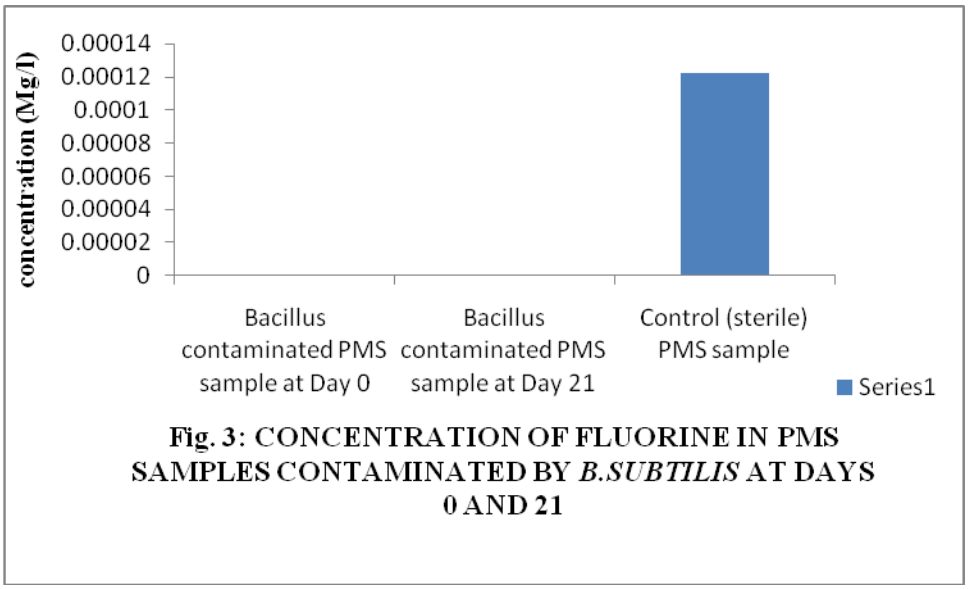

Fig. 3: CONCENTRATION OF FLUORINE IN PMS SAMPLES CONTAMINATED BY *B.SUBTILIS* AT DAYS 0 AND 21

The results from this study further validates many previous reports that have shown that bacteria species such as *Pseudomonas aeruginosa, Clostridium sporogenes, Bacillus subtilis, Aerococcus viridians, Staphylococcus aureus and Mycobacteria* spp are all indeed capable of growing in PMS (Odier, 1976; Ferarri *et al*., 1998; Gaylarde *et al*., 1999, Murygina *et al*., 2005 and Stanley *et al*., 2016) mainly because of their ability to utilize the components of the PMS as a source of food for their own metabolic processes (Ferarri *et al*., 1998; Habe and Omori, 2003; Obahiagbon *et al*, 2014). When monitored, the growth pattern of these microorganisms in petroleum hydrocarbon compounds has been shown not to be steady but wavy or fluctuating probably due to the difference in growth rates of each of the bacteria species with each bacteria attaining a maximum growth peak and declining after some days, probably as a result of exhaustion of nutrients and release of toxic material in the medium (Adekunle, 2007).

Polyaromatic Hydrocarbons (PAH) are ubiquitous in the environment (air, water, soil, sediments, tarred roads, Forests etc) (Okoro, 2008), and have been reported to be the most recalcitrant component of petroleum hydrocarbon to microbial degradation either by the aerobic or anaerobic microbial species (Heath *et al*., 1997). Results from this work align with the position of earlier workers (Das, 1991) who reported some bacteria species in association with PMS in storage. Das (1991) also noted that these bacteria were the major hydrocarbon-spoiling organisms, forming slimy mat at the bottom of the storage tank, thus causing further degradation. In view of the well-known consequences of the deterioration of petroleum compounds by microbial species on the economy, environment and people, combined with the known fact that the poly aromatic components of these compounds are more resistant to microbial attack than all its other components.

Conclusion

The results of this study revealed that bacterial species with biodeterioration potentials abound in premium motor spirit. To store them longer without adequate control-spoilage measures in place will result in their bio-attack and subsequently lead to the breakdown of motor engines and also poor action of the PMS. It is therefore recommend that adequate measure should be taken in order to reduce to the barest minimum the sources of microbial contamination on PMS in storage tanks of filling stations. Storage tanks undergrounds should have durable special coatings to prevent water from entering them; this is because water has been observed by many researchers to be an important prerequisite for development of microbial cells in fuels and oil and aid their subsequent deterioration. The results obtained in the present study is a pointer to the underlying fact surrounding the quality of the PMS that are dispensed to the public. This therefore calls for more deliberate attempts at investing resources aimed at further understanding and countering this problem.

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