Biodegradation of Benzo(a)Pyrene by Bacterial isolates from the Intestinal Gut lining of *Bos tarus*

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***Abstract*: Crude oil together with its fractionated products is the linchpin of economic development in Nigeria. Since its discovery the country has become quite influential in the global scale but with this feat, activities from both approved and non- approved explorative facilities have endangered the environment as its generated exudates deposited into both the soil-water matrix has evaded many biodegradation stratagem. In spite of the many treatment protocols many toxic compounds including PAHs are left in the environment with members that are deleterious to human health. Benzo(a)Pyrene a four carbon member of the PAH family is arguably one of the most toxic because it has been implicated as a leading cause of various cancers. Bioremediation involving microbial species from different ecological niches have been useful in solving the problems of recalcitrant polyaromatic hydrocarbons from the environment. Polyaromatic hydrocarbons (PAHs) are environmental pollutants that can be found on many surfaces including grasses that are often consumed by ruminants. It was therefore hypothesized that bacteria in the intestine of cow may have the potential to degrade Benzo(a)Pyrene. Bacteria isolated from the intestinal chyme of the large intestine of a healthy cow identified and screened for PAH degradation potential was used for bioremediation of Benzo(a)Pyrene. Two isolates with the highest degradation capacity after preliminary screening tests, was used to inoculate carbon free Bushnell Haas medium containing the PAHs in single and combined cultures for the degradation tests. Samples were withdrawn at intervals of three days and analyzed for bacterial growth and concentration loss of the B(a)P for 16 days. The two test isolates selected after screening and identification were *Escherichia coli* and *Klebsiella pneumoniae.* HPLC/GCMS analyses showed that the concentration of Benzo[a]Pyrene declined by 84.8%,91.04% and 96.44% by *E. coli, K. pnuemoniae* and a combination of both respectively after 16 days. ANOVA confirmed significant differences in the extent of the degradation of the PAHs by the test bacteria and their combined cultures ($P<0.05$). The growth of the isolates combined peaked at 1.98 log cfu/ml between days 10 and 13 during degradation of B(a)P. Phthalate was the major degradation product in the course of degradation of B(a)P. *E. coli* and *K. pneumoniae* were identified by 16S rRNA. It can be concluded that the intestine of *Bos tarus* harbor strains of bacteria that are capable of a high degree of degradation of B(a)P and *E. coli* and *K. pneumoniae* can work synergistically for bioremediation of PAH-polluted environment.**

***Keywords*: PAH, *Bos tarus*, biodegradation Benzo(a)Pyrene**

Introduction

The human populace in the bid for survival transforms the gifts of nature and by that action the environment is distorted creating newer challenges for man. It was generally thought and felt that the world was so vast that it could not be permanently affected by elemental mixtures (Kim *et al*. 2013). However, with increased industrialization, widespread pollution of the earth has become a reality leaving Homo sapiens at the mercy of diverse health challenges. These mixtures are chemically stable and resist indigenous microbial attack: natures defense mechanism. Posing serious deleterious effect on the environment and humans (Bhandari, 2009; Hadibarata and Teh, 2014).).

Benzo(a)Pyrene have been listed as a Priority pollutant because of its effects on man, the environment and indeed other living organisms. Benzo(a)Pyrene is a Polycyclic aromatic hydrocarbon (PAH) made up of carbon and hydrogen ($C_{20}H_{12}$) conjoined by fused aromatic rings in angular arrangements without hetero atom or cohere substituent (Masih and Lal 2014: Hussein and Mona 2016: Kim *et al.*, 2016).

Benzo(a)Pyrene (B(a)P) a component of crude oil and its allied products including many other factories that exude PAHs and involved in incomplete combustion has been identified as a leading cause of many types of cancer (Lawal, 2017). Thus efforts are being deployed with a number of strategies to reduce to the barest minimum the effect of B(a)P on both humans and the environment. Biotic and abiotic processes, such as volatilization, adsorption, photolysis, chemical oxidation and microbial degradation have been employed to handle the menace of Benzo(a)Pyrene. Among them, microbial degradation is a choice for consideration as it involves microorganisms from diverse sources used to remodel PAHs to less hazardous or nonhazardous compound. its activities are geared to remove or reduce toxicants in an environmentally affable and cost effective form. They in addition decrease the carcinogenicity, mutagenicity and teratogenicity of Benzo(a)Pyrene in the environmental matrices restoring the integrity of the environment (Zhao *et al*., 2022)

This research swas designed to carry out bioremediation of B(a)P using bacteria isolated from the unique semi anoxic intestinal gut lining of *Bos tarus. Bos tarus* exist in the polluted environment and feed on grasses polluted with many toxicants including B(a)P, survive amidst the pollutants because the large intestine of *Bos tarus* is packed with microscopic organisms and enzymes possessing the special ability to digest cellulose and other chemical substances (Natsuga *et al*., 2014: Nigam, 2019)

Microorganisms isolated from animal wastes and other composted materials have shown great potential in the degradation of PAHs. However, there is paucity of information on the use of microorganisms isolated from the unique semi anoxic ruminant intestinal gut environment of *Bos tarus* for the degradation of PAHs. It was therefore a plausible thought that bacterial isolates from the large intestine having survived the rigors of metabolism from the mouth of the cow through the stomach to the large intestine may be capable of degrading or metabolizing B(a)P.

Materials and Methods

Sample collection

Intestinal chyme was aseptically collected from the sigmoid colon of a male cow immediately after slaughter at the Effurun abattoir (latitude and 5.544230 longitude 5.760269, Delta state using alcohol sterilized containers. The sample was transported to the laboratory in a sterile bottle packed with ice for further use.

Physicochemical characterization of the Intestinal chyme

The pH and temperature of the intestinal chyme were determined at the point of collection of sample using hand held pH meter and in the laboratory using a Hanna Instrument's multi-parameter (HI9829). Each measurement was done three times and the average determined. All standard procedures were followed according to the manufacturer's instructions.

Isolation and purification of microorganisms

Bacteria isolation from the intestinal chyme was carried out by serial dilution method, enrichment method and direct plate methods (Avishai and Charles, 2014). **For serial dilution method**, 1g of the sample was inoculated into test tube (1) containing 9ml of distilled water (stock) and serially diluted to test tube (6). 0.1ml from the serially diluted mixture was plated on nutrient agar and tryptic soy agar plates. All cultures were incubated for 24h at 37^{o}C. The bacterial colonies, which surfaced on the plates, were carefully collected and purified by sub-culturing unto fresh agar plates using the streak plate technique. Unique colonies were selected based on the colony morphology: the appearance, opacity, coloration and texture of each isolate observed.

For enrichment method, a modified shake flask method of Siddique *et al*., (2003) as well as Fulekar *et al*., 2017 was adopted for the B((a)P spiked enrichment and bacterial isolation technique. A loopful of the stirred intestinal chyme was aseptically inoculated into 100ml sterilized MSM broth with composition (0.04 $CaCl_2{\cdot}H_2O$; 0.1 KH_2PO4; 0.8 NaCl; 1.0 NH4Cl; 0.2 $MgSO4{\cdot}7H_2O$; 0.1 KCl. Micronutrients used were (mg/L) 0.1 $CoCl_2{\cdot}6H_2O$; 0.425 $MnCl_2{\cdot}4H_2O$; 0.05 ZnCl2; 0.015 $CuSO4{\cdot}5H_2O$; 0.01 $NiCl2{\cdot}6H_2O$; 0.01 $Na2MoO4{\cdot}2H_2O$; 0.01 $Na2SeO4{\cdot}2H_2O$. and pH adjusted to 7) spiked with 100 mg/l of solid B(a)P dissolved in acetone, prepared separately in a conical flask. The mixture was incubated in a rotatory shaker for 7 days at 120 rpm, 37^{o}C. About 5ml aliquots was asceptically collected and inoculated unto a fresh 100ml sterile MSM broth, supplemented with same amount (100 mg/l) of B(A)P and incubated under same condition as reported. After four successive meliorations, the mixture was inoculated on the MSM agar plates containing thin layers of B(a)P to obtain the enriched consortium of possible PAHs degrading microorganisms identified by observing a clearing zone around the inoculated region. The isolates obtained were then purified via repeat streaking on tryptic soy/nutrient agar plates by traditional spread plate techniques to identify pure cultures. Plates were incubated at 37°C for 48 hours after which pure isolates were selected for further identification. All isolates were stored in nutrient agar slant (Zhao *et al*., 2009).

The bacterial isolates obtained from nutrient and tryptic soy agar from above were identified based on standard morphological, microscopic (Gram reaction) and biochemical tests in line with methods outlined in Bergey's manual of Determinative Bacteriology and grouped (Zhou *et al*., 2014)

Screening of isolates for the PAH degrading potential

To test the potential of isolates to degrade B(a)P, isolates were subjected to 2,6-Dichlorophenol indophenol testing adopting the method of (Roy *et al*., 2002; Syahir *et al*., 2017). A 2.5ml of carbon free Bushnell Haas medium, 150 µl of $FeCl_3.6H_2O$ suspension and 150 µl of 2, 6-DCPIP was mixed with 300 µl of bacteria standardized at (O.D. 600 nm to 0.1) and 25 µl of B(a)P (1000 mg/L in acetone). The reaction mixture was incubated at 32℃ under shaking conditions (120 rpm) for 3 days. B(a)P degradation ability of the isolates was observed by recording a discoloration of the medium from blue to colorless. A negative control was also set up containing only the mineral salt medium and the dye without microorganisms and B(a)P

The positive control contained mineral salt medium, the dye and B(a)P. The set up was left for 96hours. Colour change was monitored (Purple to colourless). The growth of each isolate was also measured using a spectrophotometer at OD_{600nm} (Youssef *et al*., 2010). Thereafter two (2) isolates with the best degradation ability were selected for further use.

Degradation of Benzo[a] Pyrene

Shake flask method (Fulekar *et al*., 2017) was adopted for degradation and the protocol for the degradation of PAHs were set as follows: sterile bijou bottles were appropriately labeled and 100µl of B[a]P in acetone was pipetted in and placed in an orbital shaker for 4 hours at 100 rpm for a final concentration of 250 mg/L using the molarity formula for calculation (molarity= mass/molar

mass) under a biosafety cabinet until the acetone evaporated completely. A 2ml of sterile carbon free Bushnell Haas media was added to each bijou bottle containing 250 mg/ml of B[a]P. Each bottle was inoculated with selected pure washed cultures and wrapped in foil (aluminum) paper before incubation in the dark in a rotatory shaker at 100 (rpm) for 16 days. Samples were withdrawn at 3 days' interval: 1, 4, 7, 10, 13, and 16 for analysis using GC-MS and HPLC

A separate set of bijou bottles containing 100µl B[a] P and 2ml sterile Bushnell Haas medium, placed under the same conditions served as a control this set up was done in triplicate. Residual B[a]P was extracted from each culture vessel using 2ml Dichloromethane. The entire content of each bijou bottle was sonicated for 15 minutes using an ultra sonicator ™. The organic layer was retrieved and evaporated in a suitable evaporator. The resulting residue was then dissolved in 2ml of acetonitrile. B[a]P was analyzed using a Shimadzu 9880 HPLC system (National Institute for Pharmaceutical Research and Development, Abuja, Nigeria). A 20µl of analyte was injected into an immobilized point capillary column measuring (15cm×4.6mm ID) and quantified by UV detector at 254nm. The glide rate of mobile phase (water and acetonitirile) maintained at 1.5 ml/min. Chromatographic peaks were matched by comparism of the retention time and spectra of control together with retention time (Wang, *et al* 2016).

RESULTS

Physicochemical characterization of the intestinal chyme

The Physicochemical characteristics of the intestinal chyme from the large intestine is presented below.

Physicochemical Characterization of the intestinal chime

Table 1

Parameter	Values
Temperature (°C)	29.5 ± 0.50
pH	7.15 ± 0.02
Moisture Content (%)	96.9 ± 0.33
Total Solids (mg/ml)	13.25 ± 0.41
Volatile solids (mg/ml)	81.26 ± 1.79

NB: All values are expressed as Mean ± SE after three replicates

Table 2**:** Morphological, Biochemical and sugar fermentation assessment of isolates from the intestinal chyme

Morphology	Microscopy	Biochemical								Sugar Fermentation						
Shape	Grams reaction	Urease	Oxidase	Catalase	Coagulase	Indole	VP	MR	Citrate	Glucose	Lactose	Sucrose	H_2S	Acid	Gas	Probable Organism
Rod	-	-	-	+	-	-	+	-	-	+	-	-	+	+	+	*Salmonella pullorum*
Cocci	+	+	-	+	-	+	+	+	-	+	+	+	+	+	+	*Citrobacter freundii*
Diplo-cocci	+	+	-	+	-	+	-	+	-	+	+	+	+	+	+	*Staphylococcus warneri*
Rod	-	+	-	+	-	+	-	+	-	+	+	+	-	+	-	*Leclercia adecarboxylata*
Cocci		+	-/+	+	-	-	+	-	+	+	+	+	-	+	+	*Micrococcus varians*
Rod	-	-	+	+		-	+	-	+	-	-	-	-	-	-	*Pseudomonas alcaligenes*
Diplo-cocci	-	+	-	+	-	+	-	+	-	+	-	-	-	+	+	*Escherichia coli*
Diplo-cocci	-	+	-	+	-	-	+	-	+	+	-	+	-	+	+	*Raoultella ormithinolytica*
Diplo-cocci	-	+	-	+	-	+	+	-	+	+	+	+	-	+	+	*Raoutella sp*
Rod	-	-	-	+	-	+	+	-	+	+	+	+	-	+	+	*Kluyvera ascorbata*
Cocci	-	+	+	-	-	-	+	-	-	+	+	+	+	+	-	*Actinobacillus minor*
Cocci	-	-	-	-	-	-	+	-	+	+	+	+	-	+	+	*Enterobacter aerogenes*
Rod	-	+	-	-	-	-	-	+	-	+	-	+	-	+	-	*Proteus penneri*
Diplo-cocci	-	+	-	+	-	+	-	+	-	+	-	-	-	+	+	*Morganella morganii*
Diplo-cocci	+	-	-	+	-	-	+	-	-	+	+	+	+	+	+	*Micrococcus roseus*
Rod	+	+	-	+	-	-	+	-	+	+	+	+	-	+	+	*Cellobiosococcus sp*

Key: - = negative, + = positive.

Table 3: Quantitative screening of bacteria by 2, 6-Dichlorophenol indophenol redox indicator using UV-VIS @600nm

Isolates	Incubation Period (h)				
	0	24	48	72	96
CTRL (+ve)	1.66±0.02[a]	1.63±0.04[a]	1.60±0.06[b]	1.60±0.06[b]	1.59±0.00[b]
CTRL (-ve)	0.29±0.05[b]	0.29±0.03[b]	0.29±0.03[b]	0.29±0.04[b]	0.29±0.01[b]
Salmonella pullorum		1.45±0.01[a]	1.16±0.03[b]	0.99±0.08[c]	0.82±0.02[c]
Staphylococcus hyicus		1.19±0.02[a]	1.17±0.06[a]	0.91±0.04[b]	0.87±0.07[b]
Staphylococcus warneri		1.25±0.07[a]	1.15±0.03[a]	0.99±0.02[b]	0.73±0.01[b]
Leclercia adecarboxylata		1.31±0.05[a]	1.16±0.03[b]	0.99±0.08[c]	0.77±0.02[d]
Micrococcus varians		1.25±0.04[a]	1.17±0.03[b]	0.93±0.02[c]	0.74±0.09[d]
Pseudomonas alcaligenes		0.97±0.03[a]	0.65±0.03[b]	0.23±0.02[c]	0.23±0.01[c]
Klebsiella oxytoca		0.99±0.00[a]	0.87±0.06[a]	0.56±0.04[b]	0.21±0.07[c]
Escherichia coli		0.87±0.04[a]	0.56±0.03[b]	0 .49±0.08[c]	0.11±0.02[d]
Raoultella ormithinolytica		0.65±0.02[a]	0.49±0.01[a]	0.30±0.01[b]	0.10±0.01[c]
Raoutella sp		0.88±0.02[a]	0.65±0.03[b]	0.23±0.02[c]	0.17±0.01[d]
Kluyvera ascorbate		0.99±0.03[a]	0.87±0.06[b]	0.71±0.04[c]	0.77±0.07[c]
Actinobacillus minor		1.14±0.04[a]	1.09±0.03[a]	1.05±0.06[b]	0.99±0.09[b]
Enterobacter aerogenes		1.24±0.08[a]	1.21±0.01[a]	0.98±0.07[b]	0.99±0.09[b]
Buttiauxella agrestis		1.01±0.01[a]	1.00±0.09[b]	0.98±0.07[b]	0.99±0.09[b]
Proteus penneri		1.03±0.02[a]	1.01±0.00[a]	0.99±0.04[a]	0.96±0.08[b]
Kluyvera cryocrescens		0.99±0.01[a]	0.87±0.06[b]	0.71±0.04[c]	0.77±0.07[c]
Cellobiosococcus sp		090±0.07[a]	0.84±0.09[b]	0.78±0.07[b]	0.76±0.09[c]

[abcd]Values across the table with similar superscripts are not significantly different at 5% based on ANOVA

Degradation of Benzo[a]Pyrene by bacteria isolates and in consortium

After calibration to obtain the line of best fit and following the sampling procedure, analysis via HPLC targeting specifically benzo[a]pyrene using selected pure cultures of *E. coli* and *K. pneumoniae* individually as well as in combination yielded results shown in table 4 Within the first day, {225, 217 and 128} mg/L was the residual concentration calculated from the peak area of B[a]P per sample vessel in comparison to initial 250mg/L concentration assayed. There was a steady decline in the residual B[a]P from day (4) four till day (16) with residual concentration 37.80, 22.41 and 8.90 for *E. coli*, *K. pnuemoniae* and the consortium respectively.

Diagrammatic representation of the percentage degradation is given in Figure 1. Data obtained from the Figure revealed a 71%, 86% and 94% degradation of B(a)P at day (16) sixteen for isolate designated *E. coli*, *K. pnuemoniae*, and consortium respectively.

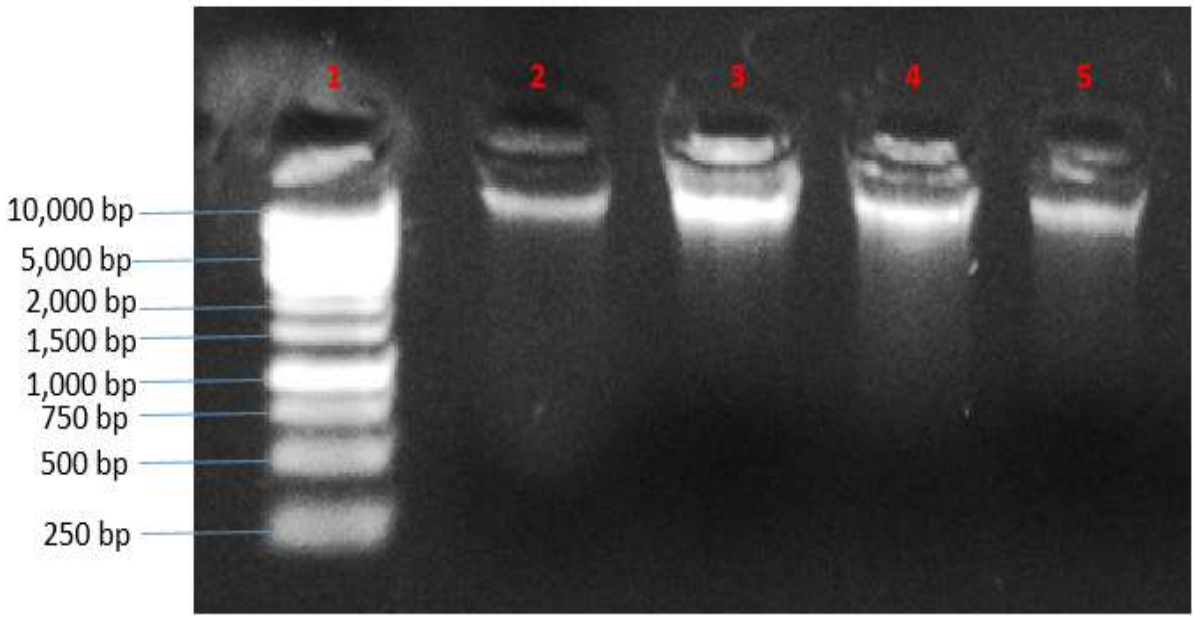

Plate 4.1: Gel Electrophoresis Pictograph of the selected bacteria isolates

Lane 1=GeneRuler™ 1kb DNA ladder (Thermoscientific). Lane 2=Isolate EC1. Lane 3=isolate EC2. Lane 4=isolate RO1. Lane=isolate RO2.

(EC1 and EC2 = *Escherichia coli* run in duplicate. RO1 and RO2 = *Raoultellaormithinolytica*run in duplicate).

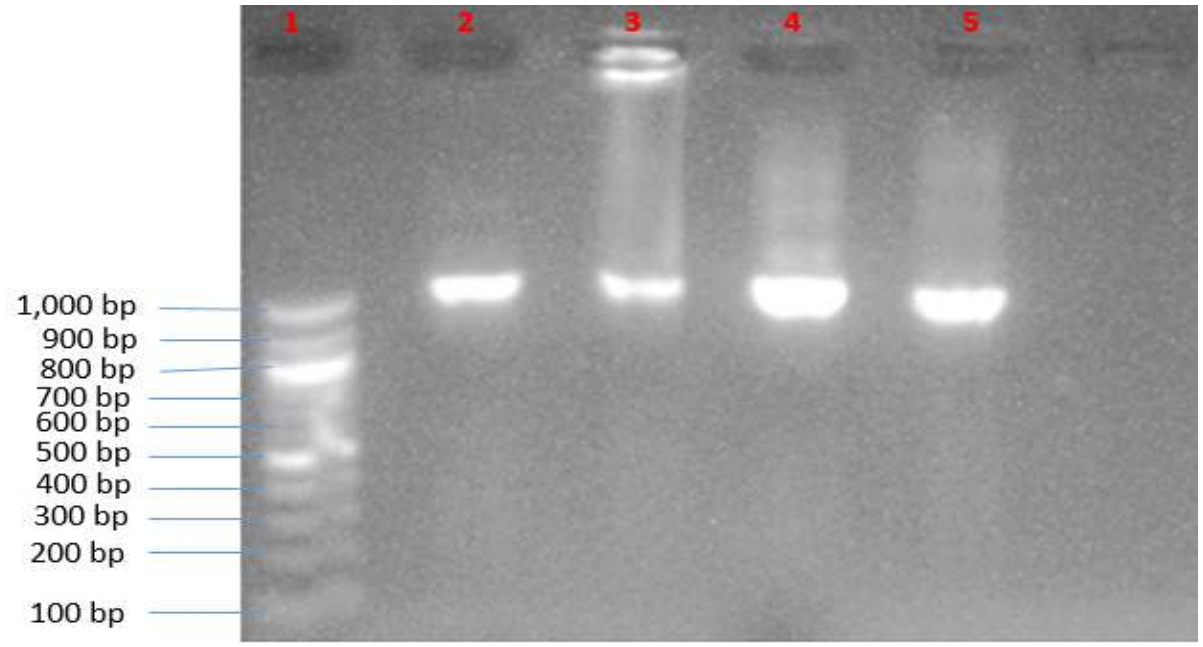

Plate 4.2 PCR analysis of the selected isolates using universal bacterial primers (27F/1492R).
Lane 1= 100bp DNA ladder (Genbiotech). Lane 2=Isolate EC1. Lanel 3=isolate EC2. Well 4=isolate RO1. Well 5=isolate RO2. (EC1 and EC2 = *Escherichia coli*run in duplicate. RO1 and RO2 = *Raoultella ormithinolytica* run in duplicate)

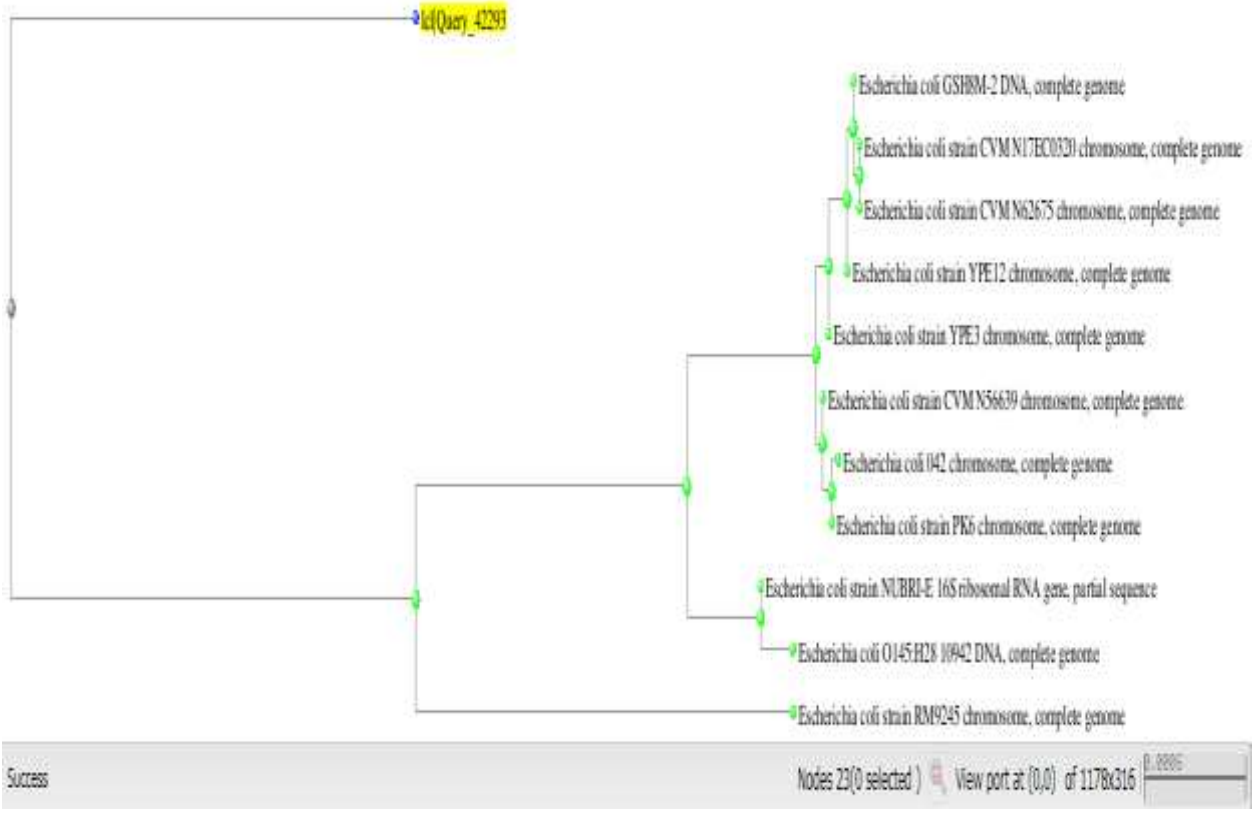

Plate 4.3**.** Phylogenetic tree of *Morganella morganii* Closest relative with 98.97% sequence similarity is *Escherichia coli* strain GSH8M-2

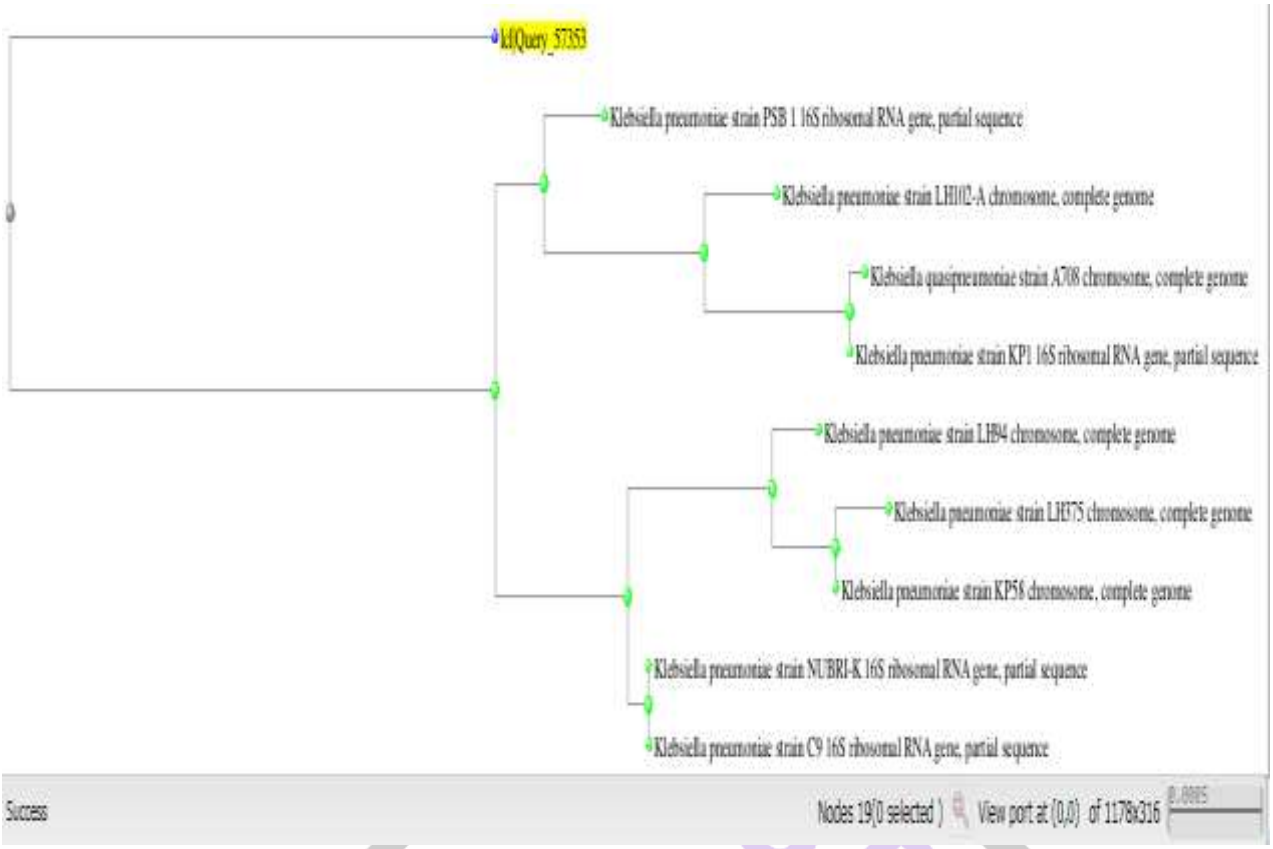

Plate 4.4 Phylogenetic tree of isolate *Raoultella ormithinolytica* Closest relative with 99.30% sequence similarity is *Klebsiella pneumoniae* strain PSB1

Table 4: Concentration of B(a)P in (mg/L) during degradation by bacterial isolates

Time (d)	Control	*E.coli*	*K. pnuemoniae*	*E. coli + K. pnuemoniae*
1	249.41±0.63	234.52 ± 0.72	214.43 ± 0.26	219.25 ± 0.99
4	230.55 ± 0.21	191.82 ± 0.55	123.53 ± 0.48	114.83 ± 0.11
7	229.74 ± 0.49	97.48 ± 0.24	88.53 ± 0.21	62.31 ± 0.01
10	222.71 ± 1.08	59.46 ± 0.36	47.6 ± 0.30	44.36 ± 0.17
13	222.13 ± 0.48	54.94 ± 0.03	36.47 ± 0.07	27.1± 0.10
16	221.25 ± 0.99	37.80 ± 0.43	22.43 ±1.56	8.90 ±0.58

*Initial concentration =250; P<0.05

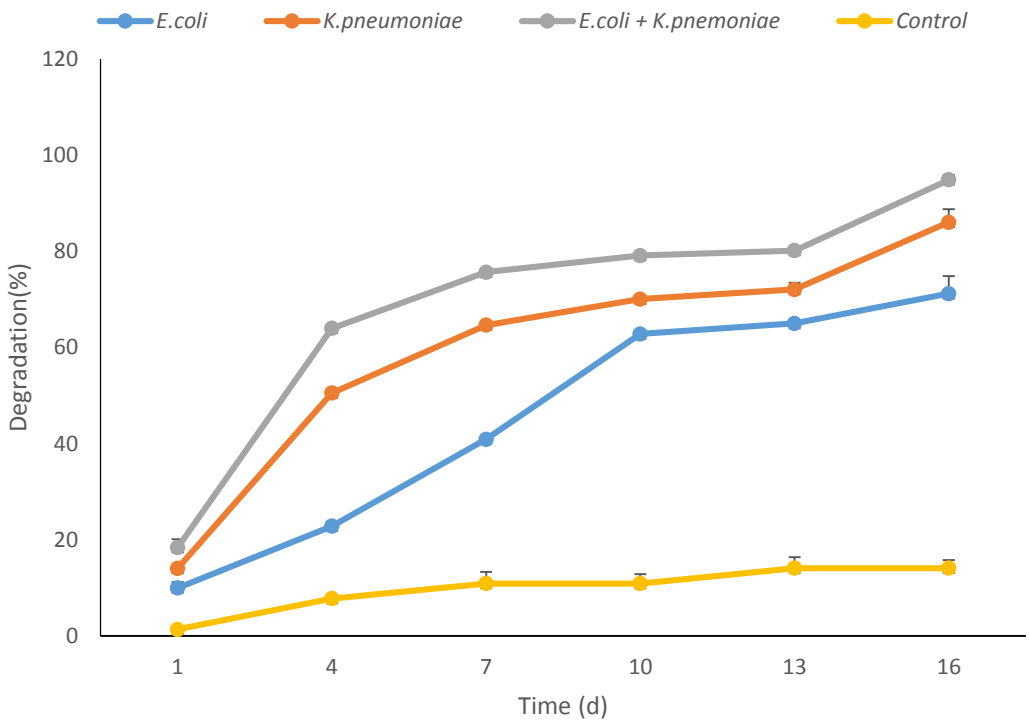

Figure 4.1: Degradation Percentage of B[a]P by *E. coli*, *K. pneumoniae* and consortium

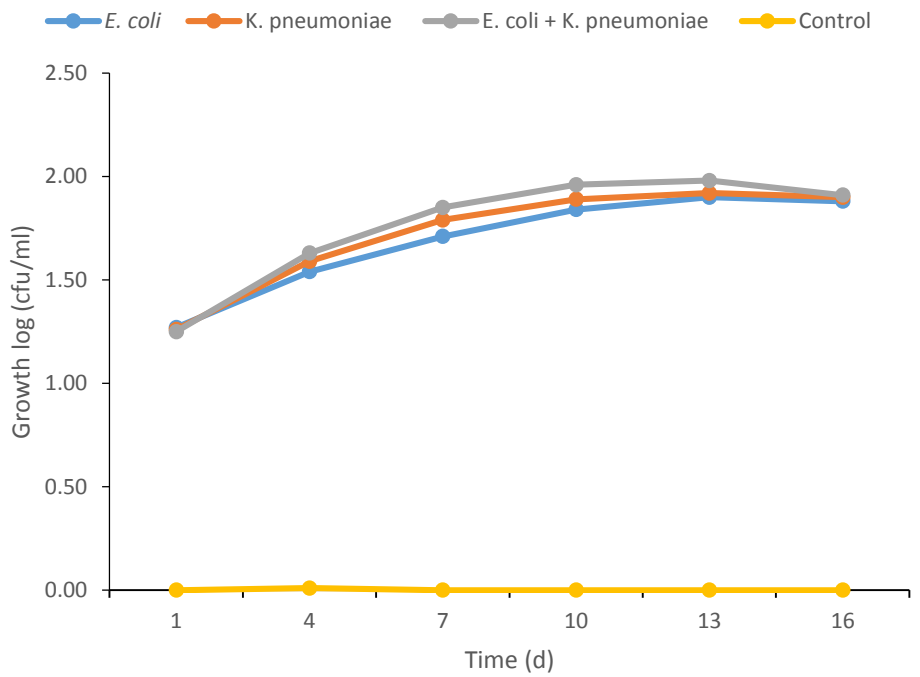

Figure 4.2: Growth of bacterial isolates during degradation of B[a]P

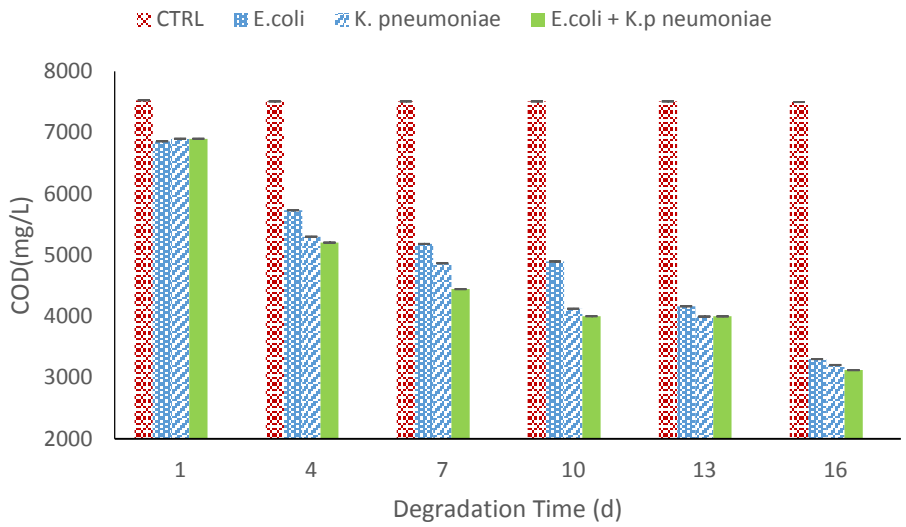

Figure 4.3: Changes in Chemical Oxygen Demand (COD) during degradation of Benzo(a)pyrene by bacteria isolates

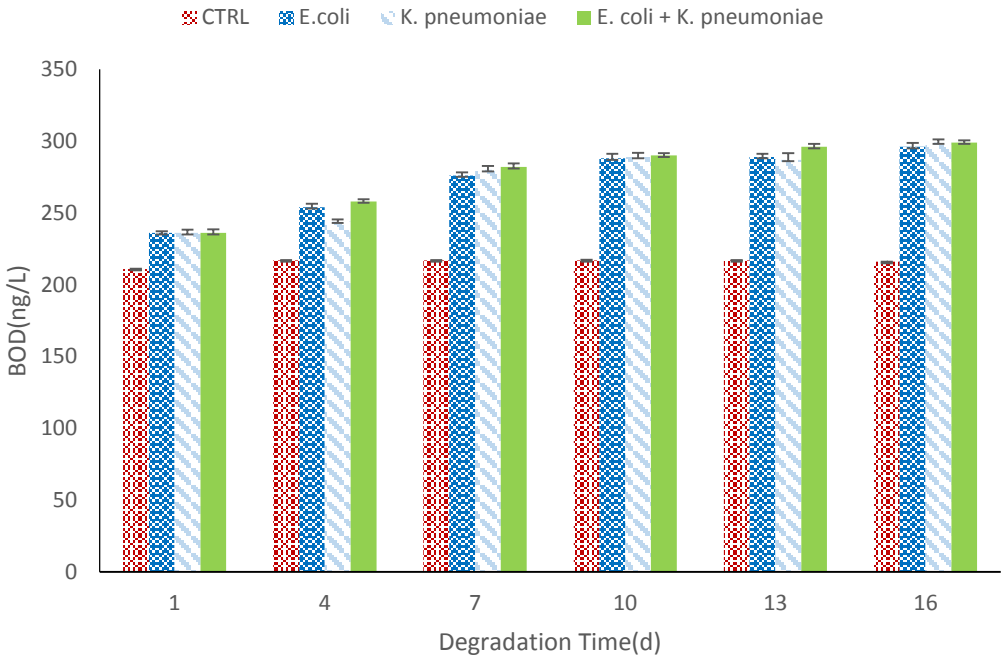

Figure 4.4: Changes in Biological Oxygen Demand (BOD) during degradation of Benzo(a)pyrene by bacteria isolates

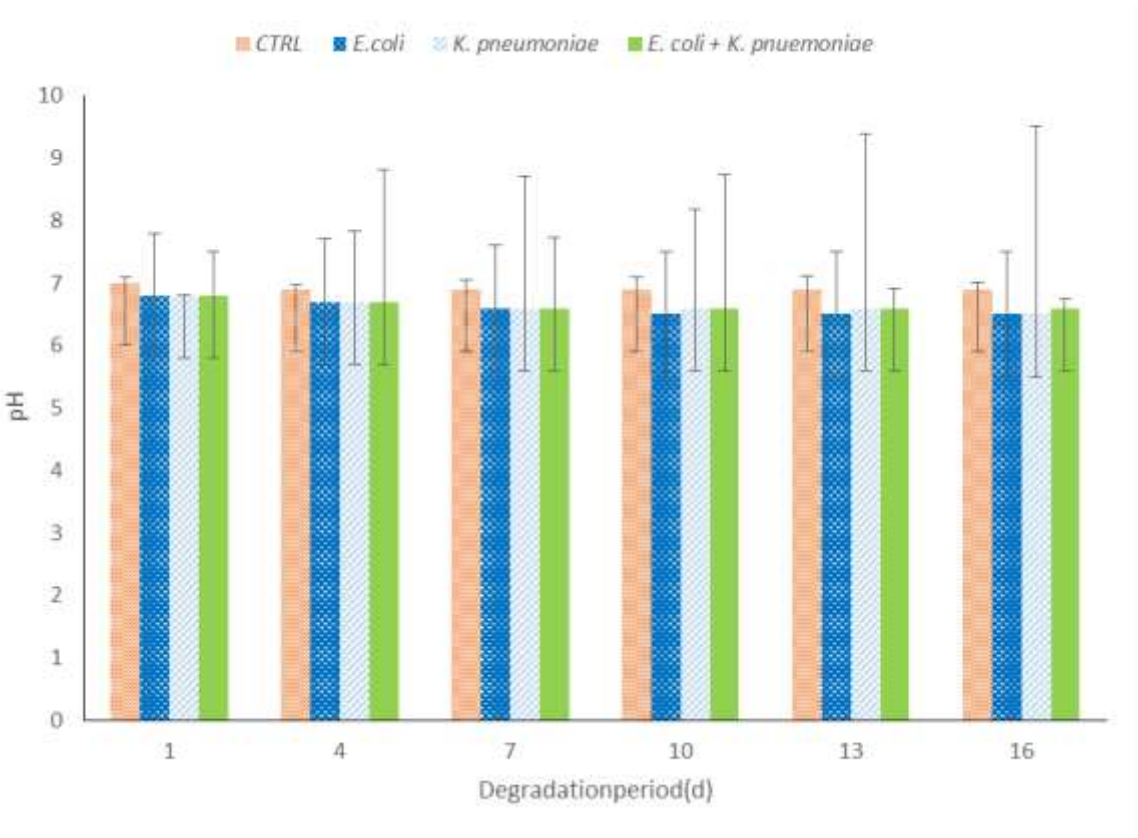

Figure 4.5: Changes in pH during degradation of Benzo(a)pyrene by bacteria isolates

Discussion

Microorganisms are ubiquitous with the inherent ability to flourish within different ecological niches where they are exposed and are continuously bombarded by an array of organic as well as inorganic compounds. Such exposure, coupled with a cascade of intrinsic metabolic biomolecules, boosts their ability to assimilate and metabolize diverse range of substances towards ensuring growth and survival (Deepa *et al.*, 2018).

The microbiota obtained from the intestinal chyme of *Bos taurus* in this study was diverse due to the rich array of materials that they consume, as well as the highly nutritional and moist environment of the large intestine which supports the propagation of several hundred species of microorganisms (Kim *et al.*, 2011). Experimental procedures have been published on the use of cow dung and other composted materials for remediation of PAHs including works of Neethu *et al*., (2019). However, little is known about the PAHs degrading microorganisms from the unique semi anoxic intestinal gut environment of *Bos taurus* which undergoes several metabolic processes including breaking down of cellulolytic materials and other conversion of chemical products serving as pollutants including PAHs (Khafipour *et al.*, 2009: Chow *et al.*, 2010). Undeniably, leaves tainted by pollutants including PAHs serve as a discrete measurement for environmental stress (Loppi *et al*., 2015) and forms the major food products for *Bos tarus*.

The microorganisms obtained in this study were members of the autochthonous microbial community which act via symbiosis to digest a wide range of vegetation for the nourishment of the vertebrate herbivores. The result in (table 1) implied that the environmental niche of the cow where the intestinal chyme used in this study was collected possessed a near neutral pH 7.15. This is line with the pH range of the intestinal gut lining of cows (6.6-7.8) Jane-Parish (2017) who explained that as materials leave the stomach they pick free radicals together with moisture and become less acidic.

The large intestine had high moisture content of between 96-98%; an ideal growth environment for the mass propagation of diverse microorganisms. This is in line with findings of (Kamra, 2005) that 90-98.5% of the fluid in both the small and large intestine of cows are moisture laced. The pH and moisture content from the ecological niche of *Bos tarus* is thought to be maintained throughout the lifespan of the host animal with slight increment in either the acidity or alkalinity contemplated due to cloistered activities of organic acids via the digestive process, coupled with splintering gaseous exchange events to restore carbon dioxide and methane balance. The average moisture content from the rumen of the studied animals aligned with data obtained from other research findings by a consortium of scientists globally (Zeng *et al*., 2019).The total solids (TS) obtained from the rumen fluid was moisture laced, which upon drying was expected to display a moderate to high percentage level owing to the fact that the large intestine represents the go-between the anus and small intestine whereby bioconversion activities take place and the end point of metabolic activities before unused and undigested food product is egested out.

In this study targeting possible aerobes or facultative anaerobes the direct plate/enrichment methods displayed a lot of similar isolates screened out for their similarities (morphorlogical and biochemical tests). The results displayed a variety of species both hydrocarbon and non-hydrocarbon degraders alike. It is conceivable that exposure to any pollutant would trigger the rise of more dominant resistant microorganisms within the same ecological niche as animals have a strong defence mechanism against any foreign body.

From the biochemical assessment of the pure cultures obtained in this study a good number of the identified bacteria belong to the Enterobactericae family probably due to the fecal proponents of the environment from which the isolates were drawn. Within the last decade, species of Enterobactericeae have been found to be prominent heterotropic petroleum degraders including PAHs, particularly the asphaltene fractions of crude oil (Patel., 2014; Subramanian & Menon, 2015; Mohamed., 2015). The results from this study also align with published data obtained in other reports like that of Sari and co-workers who isolated and positively identified *Enterobacter* species from rumen of cattle (Sari *et al.,* 2017). Many species in this genus (Enterobactericae) have adapted to exposure to a combination of extrinsic (temperature, relative humidity), implicit (microbe-microbe interaction, influence of peculiar feedstock) and intrinsic (acidity, moisture content) factors. Another visible family dominating the intestinal chyme sample from the large intestine was the Staphylococcaceae family (Table 2). Numerous species of *Staphylococcus* have been isolated from cows both dairy and beef species (Keefe, 2012; Kateete *et al.,* 2013; De Visscher *et al.,* 2014; Boss *et al.,* 2016; Capra *et al.,* 2017; Srednik *et al.,* 2017). Other families that surfaced was the Micrococcaeae and Streptoccaeae which have been implicated to be found in the intestinal lining of *Bos tarus*.

The isolates were screened in a medium containing a redox dye 2, 6-Dichlorophenol Indophenol (DCPIP) to select the best hydrocarbon degrader. DCPIP is considered to be the most reliable, cheaper and less tasking of all other screening methods for hydrocarbons (Desai and Vyas, 2006). This approach has also been described as both a quantitative and qualitative indicator method whereby upon metabolism of the hydrocarbon in the broth mixture, a colour change from blue to colourless; triggered by oxidation or transfer of electrons to one of several electron acceptors including but not limited to carbon dioxide (Grishchenkov *et al*., 2000; Boopathy, 2002 and Massias *et al*., 2003).

Data generated revealed that the best hydrocarbon degrading microorganisms using the DCPIP were probable isolates from biochemical's designated *Escherichia coli* and *Raoultella ormithinolytica*. These organisms have long been known for their petroleum degradation abilities, particularly when assayed using the DCPIP protocol (Roy *et al.,* 2002; Joshi and Pandey, 2011; Patil *et al.,* 2013; Adegbola *et al.,* 2014; Selvakumar *et al.,* 2014).The suspected *Escherichia coli* was amongst several genera of bacteria isolated from various environments by other researchers who found it capable of metabolizing polycyclic aromatic hydrocarbons; naphthalene utilized as carbon solely (Nnamchi *et al.,* 2006; Pizzul *et al.,* 2007). In their studies, they found that *Escherichia coli* exhibited a high response to utilization of crude at 600nm via a UV-VIS spectrophotometer (Nnamchi *et al.,* 2006; Pizzul *et al*., 2007). Similar finding was more recently reported by Subramanian and Menon, as well as Wanjohi and co-workers in Kenya Subramanian and Menon, 2015; Wanjohi *et al.,* 2015).

Raoutella genus of bacteria is commonly isolated from oil polluted sites and have demonstrated excellent petroleum degrading capabilities when activated (Nwaguma *et al.,* 2016). Interpretation of the Absorbance reading of data obtained from the DCPIP assay suggests that isolates obtained using mineral salt medium spiked with BaP where better metabolizers of hydrocarbons than their non-PAH-spiked variants. From the result of DCPIP UV-VIS analysis the best two hydrocarbon degraders were selected and subjected to a more robust identification method via the molecular 16SrRNA testing.

Molecular identification uses the DNA of isolates and presents an undisputed option for identification of microorganisms. Following the purification, concentration and sequencing of the amplified PCR products, raw nucleotide sequences exported from the sequenced-chromatograms using the BioEdit software followed by (BLAST) software database determined sequence similarities with already known organisms within the NCBI archive. The results showed that isolate *E. coli*, suspected to be E.coli via biochemical test analysis shared a 98.97% sequence homology with *Escherichia coli* strain GSH8M-2 (plate 1). The results showed that *Raoutella orminolytica* suspected to be *Raoutella orminolytica* based on biochemical test analysis shared a 99.30% sequence homology with *Klebsiella pneumoniae* strain PSB1 (Plate 2). Studies have revealed that the *Raoutella* family of microorganisms consists predominantly of Gram-negative, oxidase negative, non-motile, capsulated, aerobic and facultative anaerobic rods, which originated from the *Klebsiella* family only to be redefined and separated based on advanced recombinant DNA techniques (Drancourt *et al.,* 2001). Both *Raoutella* and *Klebsiella* belong to the Enterobacteriaceae super family with the major difference being the presence of the *rpoβ* gene in the former (Ponce-Alonso *et al.,* 2016).

Escherichia coli(S13) and *Klebsiella Pnuemoniae(PSB8)* showed themselves strong in the degrading (B[a]P. The pure compound after preparation and standardization was quantified using HPLC to establish a peak and retention time together with recovery limit of detection (LOD) and limit of quantification(LOQ). Researches have shown that the response of microorganisms to pollutants in a medium is varied and is dependent on the type and concentration of the pollutants (Gauri *et al*., 2019). Data obtained from HPLC analysis of (B[a]P) showed that isolates from the large intestine had the capacity to reduce (B[a]P) although at different rates. *Klebsiella pnuemoniae* exhibited a higher degradation efficiency (14.1%) than *Escherichia coli* strain (9.9%) and a combination of both isolates of the initial 250 µg/ml of B[a]P after 24 hours on incubation (Table 4). While the initial kick off after 24hrs saw *Klebsiella pnuemoniae* performing better than *E. coli* and a combination of both isolates the pick-off of the isolates as degradation went on showed the synergy in which *E. coli* and *K, pnuemoniae* have working together to attain a 94.8% degradation better than the 71.2% for *E. coli* and 74.8% after sixteen days. This corresponds with the findings of Nwaguma *et al*. (2016) that *E. coli* and *K. pnuemoniae* from human feaces could degrade petroleum hydrocarbon. The expectation was that the control sample will remain at the initial concentration since it was asceptically prepared without microorganisms however there was slight decrease in its concentration. This may have been due to volatization as prolonged volatilization has been implicated in reduced PAH concentrations during pilot studies reported by Kong *et al.* (2015). Whilst utilization of B[a]P for energy generation was verified in individual and mixed cultures, observed pattern showed variation in regards to their specific growth rates (Figure 1). Bacteria growth on B[a]P was relatively slow for *E. coli* the combination of *E. coli* and *K. pnuemoniae* was better though still not fantastic. Probably due to the acclimatization rates (substrate metabolism) of *E. coli* to the B[a]P substate and limited transport or cytotoxicity (Sowada *et al.,* 2014). Studies have shown that degradation of B[a]P under aerobic conditioning is usually via a stepwise oxygenation process (Cébron *et al.,* 2008). In that same report, it was deduced that bacterial utilization of weighty PAHs like B[a]P is usually achieved using mixed microbial consortiums. As observed in this study, the lower rates of PAH biodegradation by

individual bacteria opposes the energy consumption required for the complete bioconversion/bioremediation (Andersson and Hughes, 2010; Haiser and Turnbaugh, 2013)

It has been established that, the concentration of the hydrocarbon pollutants (in a controlled environment) affects the survival of microorganisms as the survival of the organism depends on the utilization of carbon from the substrate for energy. This research work agrees with the above observations. Accordingly, there was progressive growth for isolates singly and in combination. Although B(a)P was degraded but it was not completely mineralized hence there was no decrease in population as concentration decreased up until the sixteenth day.

Probably due to the fact that *K. pneumoniae* had prior exposure to the pollutant so acclimatized was quicker than its *E. coli* partner or maybe attributed to the unique enzyme cascade, crucial to both the electron transfer and bioconversion of hydrocarbon principle of which are the mono- and dioxygenase genes (*AlkB, C23O* including *PAHRHDα* as reported by many researchers (C'ebron *et al.*, 2008; Guo *et al.*, 2010). The structural system spectrum of one and multiple oxygenase enzyme play a major part in the initiation of PAHs degradation aerobically (Vaidya *et al*., 2018) thus throughout the period of the assay the dissolved oxygen level was analyzed and maintained by ensuring agitation using an orbital shaker.

Chemical oxygen demand (COD) was observed to have a direct proportional relationship with the concentration of the PAHs. The COD decreased with corresponding decrease in concentration of B(a)P in MSM. Cogent decrease in COD concentrations was observed at each sampling day till the sixteenth day. BOD on the other hand, showed an increase as concentration decreased during the assay run for the experimented PAHs showing an inversely proportional relationship with growth of the organism. This finding tallies with the research of Fulekar *et al*. (2017) who reported similar findings with lower molecular weight PAHs.

Degradation of PAHs by microorganisms depends on some physicochemical parameters, which includes: pH, temperature and concentration. In this experiment *E. coli* and *K .pnuemoniae* followed the normal growth curve pattern in which on exposure experience the period of acclimatization (lag phase) and with time may pick up to exponential phase, stationary and then decline phase. The response of the pollutant in respect to growth and degradation is dependent on some variables including microbial proliferation rate, percentage loss of pollutant including intermediaries culminating in determination of BaP reduced in a medium. The obtained result for the BaP analyzed revealed the metabolism of the hydrocarbon been dependent on the microbial number which in turn correlates to the time of exposure.

Both studied bacteria in this research work validated other published work by several scientists and adds to the knowledge base of BaP biodegraders from the unique semi anoxic intestinal gut environment of *Bos tarus*.

This data obtained pertaining to *Escherichia coli* supported reports by Kim and colleagues (2007) who found that *Escherichia coli* like other PAH degradable bacteria possesses certain resistant genes especially laccases coded by a *CueO* gene that is more predominant and widely distributed in this organism than most. The 5-ringed structure of B[a]P appear to favour the growth and metabolism of *E. coli* better than that of *K. pneumoniae*, which upon escalated expression in response to environmental stimuli, enable them to degrade certain toxic chemicals in order to prevent oxidative, membrane and protein damage, usually brought about upon prolonged exposures to PAHs simple and complex structures alike (Kim *et al.*, 2007).

Therefore, a combination of *E. coli and K. pnuemoniae* is efficient in the degradation of the PAHs used in this study and should be considered in the remediation stratagem of contaminated environments by poly aromatic hydrocarbons. The competence developed by microorganism from enriched consortium from the rich intestinal gut of rumen showed great capability to use and reduce PAHs generating survival energy through phthalic acid reduction pathway. It also laid bare the fact that the physicochemical parameters/prevailing environmental conditions have profound contributory roles in the removal or conversion of polyaromatics.

Conclusion

The findings from this research showed that *E. coli* and *K. pneumoniae* had good synergy in the degradation of BaP. These enterobacteria isolated from the intestinal gut lining of *Bos tarus* suggested that the microbiota from the large intestine possess a strong molecular gene cluster based on the presence of selected hydrocarbon degrading genes in the selected isolates that have been implicated for PAH degradation in organisms from varied sources. Implying that that the combination of *E. coli and K. pnuemoniae* from the intestinal gut lining of *Bos tarus* was efficient in the degradation of PAHs through the phthalic acid degradation pathway

- Intestinal strains of *E. coli* and *K. pneumoniae* were established as strong degraders of BaP however, the consortium of both isolates had the highest degradation.
- The *o-phthalate* pathway is crucial in the use and reduction of BaP.

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