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RESEARCH ARTICLE

EFFECTS OF PHYSICAL PARAMETERS AND ALGORITHM OF PETROLEUM HYDROCARBONS UTILIZATION BY SOME BACTERIAL ISOLATES FROM BONNY, RIVERS STATE NIGERIA.

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Abstract

The ability of some bacterial isolates to utilize crude oil and the effects of pH and various nitrogen sources on the growth and utilization of hydrocarbons were investigated. A total of six bacterial isolates were isolated. These include two different species of Bacilli, three different species of Pseudomonas and Klebsiella species. The effects of pH on the growth of the isolates showed that Pseudomonas sp (A2) had the highest growth of about 2.25×10^5 cfu/ml at pH 6.5. Bacillus sp (A5), Klebsiella sp (A1) recorded growths of 2.04×10^5 and 1.70×10^5 cfu/ml at pH 7.5 and 7.0 respectively. These isolates were able to utilize and grow on different nitrogen sources containing ammonium sulphate $((\text{NH}_4)_2\text{SO}_4)$, sodium nitrate (NaNO_3) , and ammonium chloride (NH_4Cl) . NH_4Cl was the best utilized nitrogen source with growth of 2.37×10^5 , 2.17×10^5 , and 1.93×10^5 cfu/ml for Pseudomonas, Bacillus and Klebsiella species respectively. The effects of pH on the utilization of Bonny light crude oil revealed percentage utilization of about 76%, 68%, and 58% at pH of 6.5, 7.5 and 7.0 respectively for Pseudomonas, Bacillus and Klebsiella species. Percentage utilization of about 67%, 63%, and 51.8% were recorded for Pseudomonas, Bacillus and Klebsiella species when grown on mineral salt media supplemented with NH_4Cl . The isolates were tested for their ability to utilize bonny light crude oil using the gravimetric method. The percentage utilization of all the isolates ranged between 77.5%, 66.8%, and 54.2% after 28 days of incubation for Pseudomonas, Bacillus and Klebsiella species respectively.

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Introduction:-

Petroleum-based products are the major sources of energy for industry and daily life. These products enter into the environment through the activities of petroleum extraction, refining, transportation, storage, bad practices (human error), accidents and leakages due to corrosion of tanks and pipelines as well as vandalization and storage of petroleum and petroleum products (Wokem et al., 2017). Soil contamination with hydrocarbons causes extensive damage of local system which may cause death or mutations (Alvarez and Vogel, 1991).

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Biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and on the amount of the hydrocarbons present. Many microorganisms have the ability to utilize hydrocarbons as sole sources of carbon as energy for metabolic activities and these microorganisms are widely distributed in nature. Microbial utilization of hydrocarbons depends on the chemical nature of the compounds within the petroleum mixture and on environmental determinants (Adeline et al., 2009). The process of bioremediation, defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry (Ulrici, 2000). Bacteria are the most active agents in petroleum degradation, and they work as primary degraders or feeders of spilled oil in environment (Brooijmans et al., 2009; Yakimov et al., 2007). It is therefore the aim of this research to investigate the algorithm of petroleum hydrocarbon utilization potentials of some bacterial isolates from oil spilled environment in Bonny, Rivers State Nigeria.

Materials And Methods:-

Study Site and Sample Collection

The soil samples were collected from an oil-polluted land in Bonny Local Government Area of Rivers State (Latitude: 4°25' and 4°50' N, Longitude: 7°00' and 7°15'E). Sample was collected directly from 5 different sites at a depth of 0-20 cm into an aluminum foil paper using sterile spatula and transported immediately to laboratory where microbiological analysis was carried out.

Total Heterotrophic Bacteria(THB)Count:

The method of Ebuehi et al., (2005) was used with slight modification. Total bacterial population in the oil-polluted soil sample was enumerated and isolated adopting serial dilution and the standard plate counts technique using pour plate method. THB was obtained by preparing serial dilution of soil samples with 10g of soil in 100 ml of distilled water and plated on nutrient agar in triplicate. Culture plates were incubated at room temperature 28°C for 48h. Plates yielding counts of 30 – 300 colonies were chosen and the counts obtained were multiplied by the dilution factor to obtain the number of bacteria per gram of soil.

Total Hydrocarbon Utilizing Bacteria(THUB) Count:

Vapour-phase transfer method was adopted to estimate the population of THUB. A modified Bushnell-Haas mineral salt agar (MSA) was used in this study comprising of 0.2 g of MgSO₄, 0.02 g of CaCl₂, 1 g of KH₂PO₄, 1 g of K₂HPO₄, 1 g of NH₄NO₃, 0.05 g of FeCl₃. One hundred mill of distilled water at pH of 7.0, sterilization was carried out at 121°C for 15 min (Sidkey et al., 2016). Soil suspension was prepared by serially diluting 10g of soil in 100ml of distilled water and plated in triplicate. Sterile filter paper (Whatman No. 1) saturated with crude oil was placed on the inside cover of each petri-dish kept in an inverted position. These filter papers supplied the hydrocarbons by vapour phase transfer to inverted inoculums. Plates were counted after incubation at room temperature for 7 days. The percentage of hydrocarbon utilizers within the heterotrophic bacteria population was determined (Ebuehi et al. 2005)

Characterization and Identification of isolates.

Bacterial isolates were characterized based on colonial and cell morphology, growth on differential/selective media and biochemical tests which include Gram's reaction, indole tests, methyl red, Voges-Proskauer, citrate utilization, Urea test, utilization of different types of sugars, oxidase and catalase tests. Pure cultures of bacterial isolates were identified on the basis of their colonial morphology, cellular morphology and biochemical characteristics according to the taxonomic scheme of Bergey's Manual of Determinative Bacteriology, as reported by Salam et al., (2011).

Screening of Isolates for Petroleum Hydrocarbon Utilization:

Bacterial isolates from the mineral salts agar were screened for utilization of crude oil. A 24 h culture suspension of each isolate was standardized and inoculated into the sterilized mineral salts broth supplemented with 20% crude oil and incubated on a shaker (150 rpm) at 28±2°C for 7 days. Development of turbidity was visually assessed as a measure of hydrocarbon utilization, and recorded as -, no turbidity; +, low turbidity; ++, moderate turbidity and +++, high turbidity. Thereafter, absorbance of the culture was measured with spectrophotometer and the optical density (OD) was recorded at 460nm for the various treatments. Isolates producing intense turbidity with visible disappearance of oil were selected and stored for further studies. (Onugbolu and Adieze, 2016)

Preparation of Standard Inoculum

Three different bacteria isolates with highest turbidity were labeled and cultured on nutrient agar and incubated for 24 h at 37°C. Then, a single colony of each bacterium was inoculated into nutrient broth and incubated at 37°C in an orbital shaker for 24 h at 150 rpm. Then, the broths were centrifuged at 4000 rpm for 15 minutes. Supernatants were decanted and pellets containing bacterial cells were centrifuged with distilled water twice to ensure removal of all broth components. The concentration of each inoculum was measured using a spectrophotometer to obtain 0.1 optical densities in 600 nm wavelength. The above standard inoculums were used in the following step reported by Kalaivani (2013).

Determination of the Effects of Physical and Nutrient Parameters on Microbial Growth and Biodegradation of Crude Oil

For all the experiment below, the following standard procedures were used.

Two percent (v/v) of standardized inoculums was inoculated into test tubes containing 24 ml of MSM supplemented with 5% (v/v) of Bonny light crude oil and incubated in an orbital shaker for 7 days. The negative control in these tests was MSM without inoculation. After 7 days of incubation, the cultures were serially diluted 6 fold using normal saline solution and the growth was estimated by plating the bacteria on nutrient agar by the spread plate technique. Residual oil was checked using gravimetric method.

Determination of Effects of pH on Biodegradation of Crude Oil

The pH of each medium was adjusted to 6.0, 6.5, 7.0, 7.5 and 8.0, with 1 M of NaOH or 1 M of HCl. The cultures were incubated at 37°C in an orbital shaker at a speed of 150 rpm.

Determination of the Effects Nitrogen Sources on Biodegradation of Crude Oil

To determine the effect of nitrogen source on the utilization of crude oil, the total amount of nitrogen in MSM which was contained in ammonium nitrate (NH_4NO_3) = 10 g/l was replaced with different nitrogen sources, namely, ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$), sodium nitrate (NaNO_3), ammonium chloride (NH_4Cl).

Crude Oil Degradation Studies.

The ability of isolates to degrade crude oil was evaluated using the gravimetric method demonstrated in terms of reduction in the weight of crude oil introduced. The rate of utilization was monitored on the first day (day zero) of the study and subsequently at 4-day interval for 28 days; n-hexane was employed as the solvent for extraction. On each day, three samples per single treatment were analyzed for the quantity of residual crude oil using the method described by Nwaogu et al. (2008) with slight modifications. Two percent (v/v) of standardized inoculums was inoculated into test tubes containing MSM supplemented with 20% (v/v) of Bonny light crude oil and incubated in an orbital shaker for 28 days. The negative control in these tests was MSM without inoculation.

Extraction of Residual Oil.

The method described by Eniola et al. (2014) was adopted. The residual oil was extracted by using liquid – liquid solvent extraction method. The organic solvent used was n-hexane. This was done by measuring 50ml of n-hexane into the bottles containing OIL-MSM, the contents were later transferred into separating funnel. A funnel fitted with filter paper (Whatman No 1), anhydrous sodium sulphate spread on the filter paper was employed to remove any moisture in the mixture, this was used to collect the layer containing the organic solvent and residual oil in a pre-weighed 50ml pyrex beaker. The organic solvent was allowed to evaporate in an oven, after the evaporation, the amount of residual oil was calculated as follows:

Weight of oil degraded = Original weight of oil minus weight of residual oil obtained after evaporating the extractant.

Percentage degradation = (Weight of crude oil degraded / Original weight of oil introduced) $\times$ 100.

Results:-**Total Heterotrophic and Hydrocarbon Utilizing Bacterial Count.**

The results obtained from the microbial isolation and identification indicates that the soil sample contained relatively high total heterotrophic and hydrocarbon utilizing bacteria with colony forming unit per gram (Cfu/g) of $1.38 \pm 0.46 \times 10^8$ and $5.84 \pm 0.23 \times 10^7$ cfu/g respectively. Hydrocarbon utilizers obtained from the soil was 42.3%.

Table 1: Total heterotrophic and hydrocarbon utilizing bacterial count.

Sample	Total Heterotrophic Count	Hydrocarbon Utilizing Count	%HC Utilizers
Soil	$1.38 \pm 0.38 \times 10^8$ cfu/g	$5.84 \pm 0.23 \times 10^7$ cfu/g	42.3

Table 2:-Morphological and Biochemical Characteristics of Bacterial Isolates.

Character	Isolate code					
A1	A2	A3	A4	A5	A6	
Microscopy	Rod	Rod	Rod	Rod	Rod	Rod
Gram stain	-	-	-	+	+	+
Motility -	+	+	+	+	+	+
Catalase +	+	+	+	+	+	+
Citrate	+	+	+	+	+	+
Oxidase -	+	-	-	-	-	-
Indole	-	-	-	-	-	-
Urease	+	-	-	-	-	-
Glucose +	-	-	-	-	-	-
Lactose +	-	-	-	-	-	-
Gas	+	-	-	-	-	-
H ₂ S	-	-	-	-	-	-
Indole	-	-	-	-	-	-
V. P	-	-	-	+	+	-
Methyl-Red	+	-	+	-	-	+
Organism	Klebsiellasp	Pseudomonas sp	Pseudomonas sp	Bacillus sp	Bacillus sp	Pseudomonas sp

Key: V.P; VogesProskauer

Effects of pH and Nitrogen sources on the Growth of the Isolates

Considering the effects of various pH ranges on the growth of bacterial isolates as presented in figure 1, all of the bacterial isolates were able to grow at various pH ranges. Psuedomonas sp showed the highest growth at pH 6.5. Bacillus sp grew best at pH 7.5 while Klebsiella sp grew best at pH 7.0. Pseudomonas sp produced the highest growth with 2.25×10^5 cfu/ml while Bacillus and Klebsiellasp had 2.04×10^5 cfu/ml and 1.70×10^5 cfu/ml respectively.

The isolates were able to utilize all types of nitrogen sources tested containing ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$, sodium nitrate (NaNO_3) and ammonium chloride (NH_4Cl). Ammonium chloride was the most utilized nitrogen source in this study with Pseudomonas sp producing the highest growth of 2.37×10^5 cfu/ml, followed closely by Bacillus sp with 2.17×10^5 cfu/ml while Klebsiellasp was 1.93×10^5 cfu/ml. Figure 2 shows the effects of various nitrogen sources on the growth of the isolates.

Effects of pH and Nitrogen sources on the Utilization of Crude Oil

The effects of these isolates on the utilization of crude oil were tested at various pH ranges and presented in figure 3. At pH 6.5, about 76% loss of oil was recorded by Pseudomonas sp. Bacillus sp recorded a 68% loss at pH of 7.5 while Klebsiellasp had a 58% loss at pH of 7.0.

The ability of the isolates to utilize crude oil supplemented with different nitrogen sources were tested and presented in figure 4. About 67%, 63% and 51.8% loss of crude oil were recorded for Pseudomonas, Bacillus and Klebsiella species respectively when supplemented with ammonium chloride. Ammonium chloride was the best utilized nitrogen source. Sodium nitrate recorded a 62.4%, 56.7% and 44.4% loss for Pseudomonas, Bacillus and Klebsiella species respectively. Ammonium sulphate was the least utilized with 53.2%, 46.7%, and 36% loss of crude oil for Pseudomonas, Bacillus and Klebsiella species respectively.

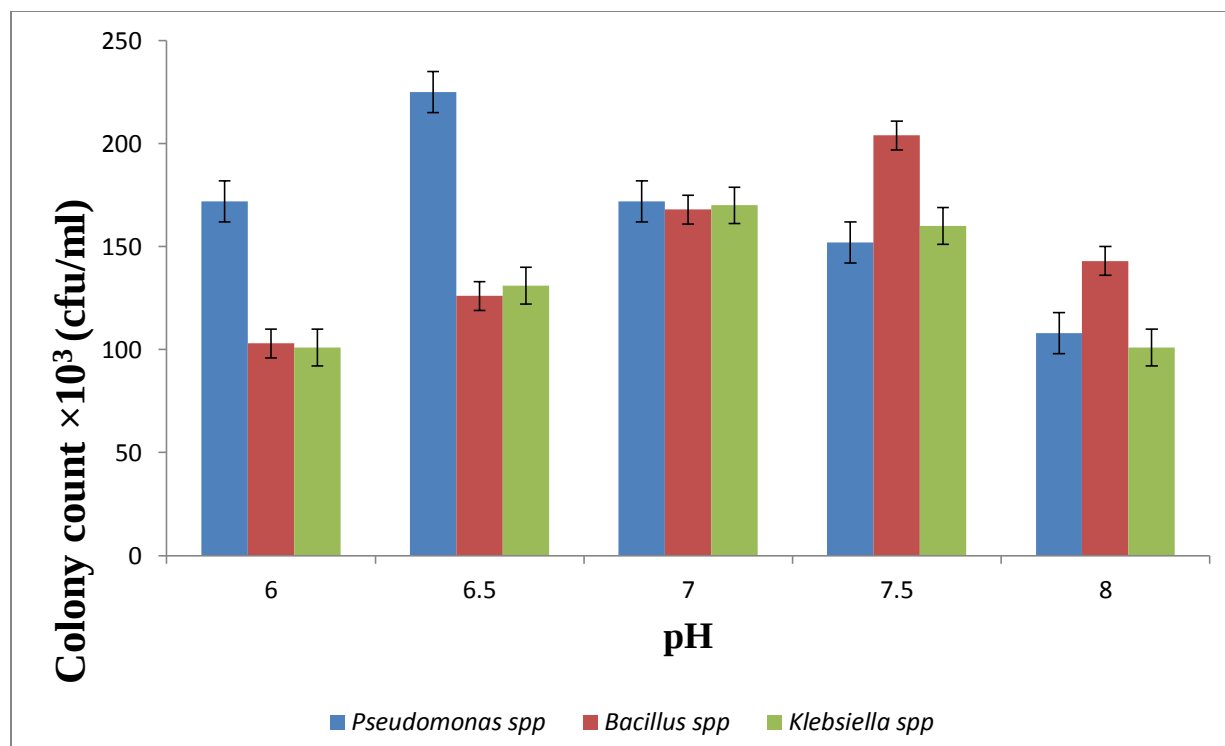


Figure 1:-Effects of pH on the growth of the isolates.

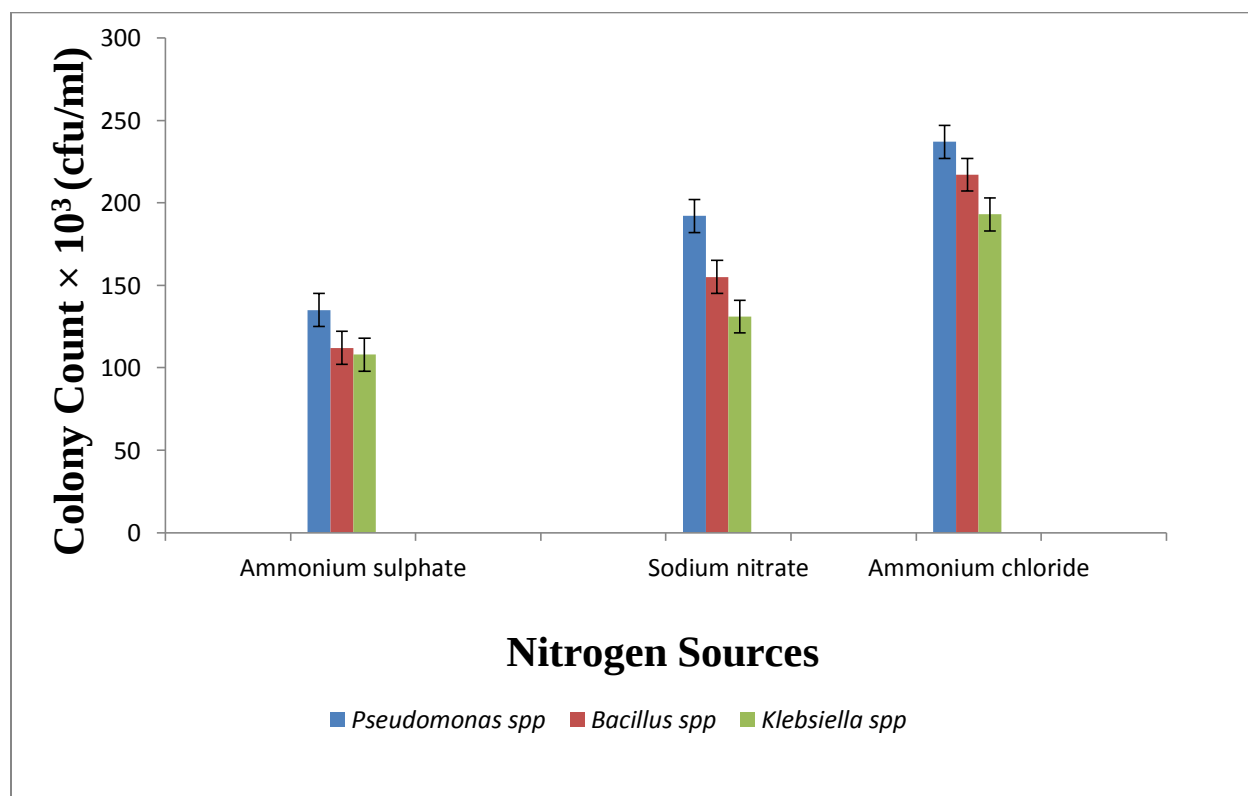


Figure 2:-Effects of various nitrogen sources on the bacterial growth of the isolates in MSM supplemented with 5% v/v of crude oil.

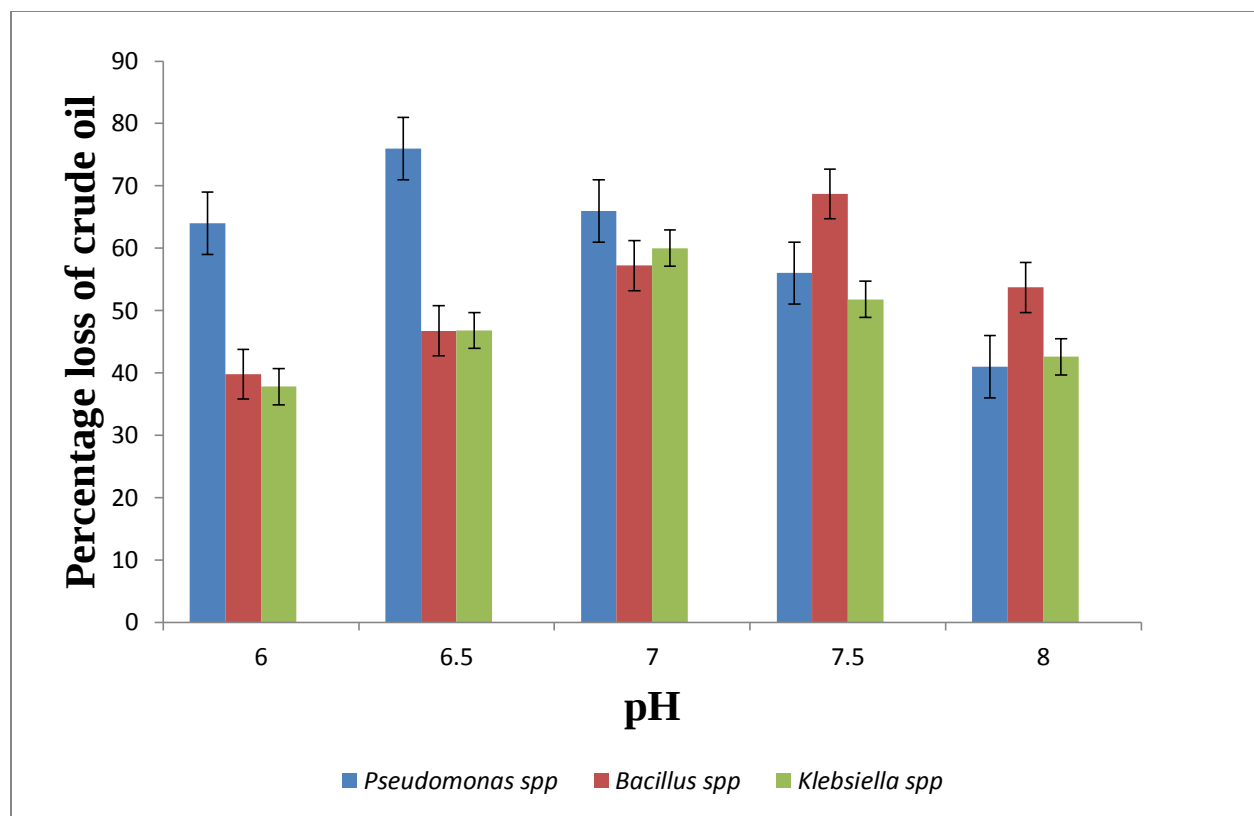


Figure 3:-Effects of pH on the utilization of crude oil by the isolates.

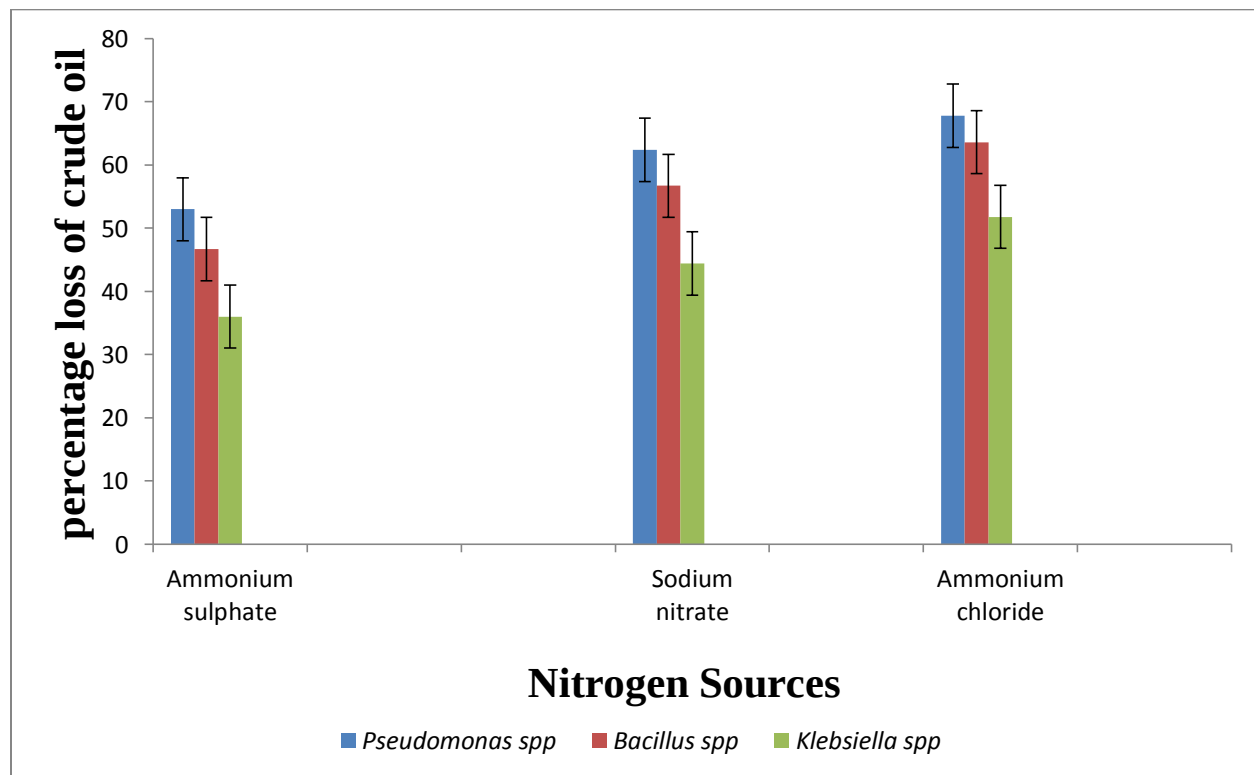


Figure 4:-Effects of nitrogen sources on the utilization of crude oil.

Crude Oil Utilization Studies

Pseudomonas, *Bacillus*, and *Klebsiella* species were used for the utilization studies and result is presented in figure 5. After 28 days of incubation, the highest oil utilization was observed in *Pseudomonas* sp (77.5%), this was followed by *Bacillus* sp (66.8%) and *Klebsiella* sp (54.2%).

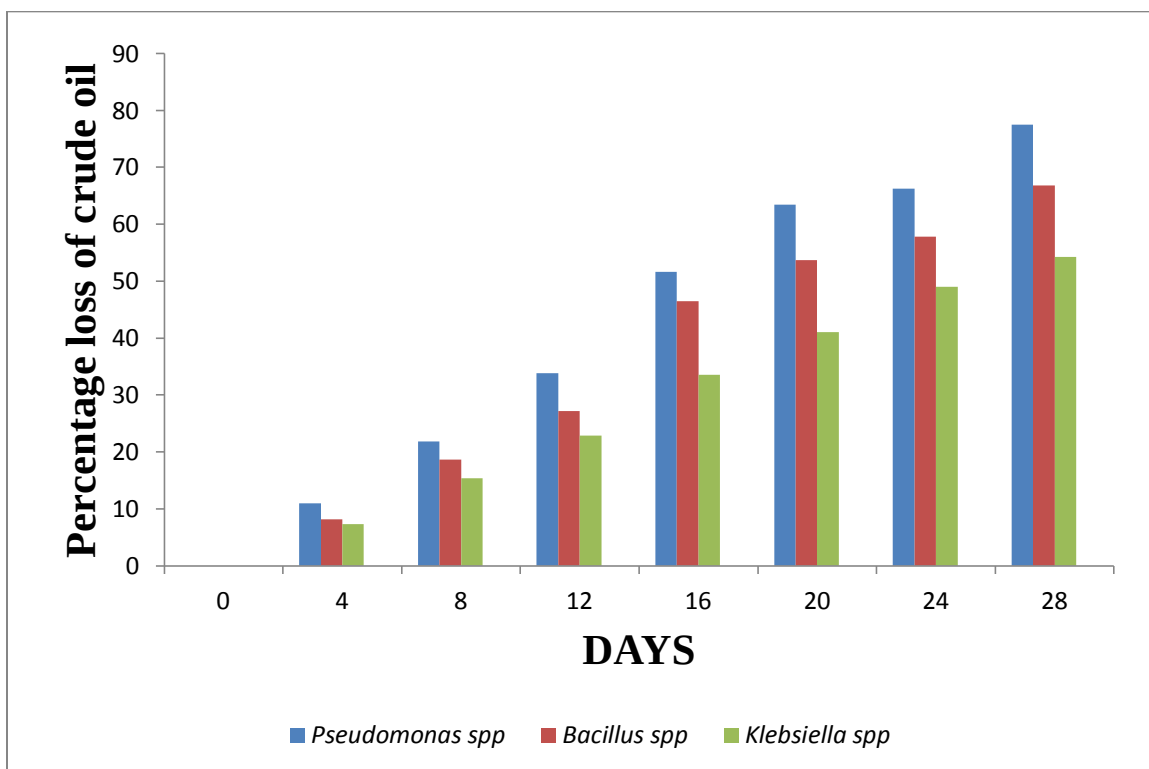


Figure 5:-The percentage utilization of crude with respect to incubation time (28 days).

Discussion:-

Total heterotrophic and hydrocarbon utilizing bacterial counts from the soil sample were $1.38 \pm 0.38 \times 10^8$ cfu/g and $5.84 \pm 0.23 \times 10^7$ cfu/g respectively. Percentage calculation showed that 42.3% of the total heterotrophic bacterial counts were hydrocarbon utilizers. This shows that bacterial species with potential ability to utilize hydrocarbons exist ubiquitously in the environment as reported by Onugbolu and Adieze (2016). Results from the morphological and biochemical characteristics of the isolates shows that a total of six bacterial isolates were obtained belonging to three genera. These include three species of *Pseudomonas*, two species of *Bacillus* and a species of *Klebsiella*.

Effects of Physical and Nutrient Parameters on the Growth of the Isolates

Considering the effects of various physical and nutrient parameters on the growth of bacterial isolates, all of the bacterial isolates were able to grow at various pH ranges. *Pseudomonas* sp showed the highest growth at pH 6.5. Hence it could be said that the optimum pH for the growth of *Pseudomonas* sp is 6.5. However, at pH 6.0 and 7.0 the growth of the isolates was the same. This indicates that pH ranges between 6.0 and 7.0 can be used for the growth of *Pseudomonas* sp. This correlates with the findings of Ainom et al., (2010) who reported the optimum pH of *Pseudomonas aeruginosa* at 6.5. Yuan et al., (2002) isolated *Pseudomonas auorescens* and *Haemophilus* sp from soil contaminated with petroleum effluent discharge that grew at optimum pH of 7.0. *Bacillus* sp and *Klebsiella* sp were able to grow at various pH ranges, with *Bacillus* sp growing best at pH 7.5 while *Klebsiella* sp grew best at pH 7.0. *Pseudomonas* sp produced the highest growth with 2.25×10^5 cfu/ml while *Bacillus* and *Klebsiella* sp had 2.04×10^5 cfu/ml and 1.70×10^5 cfu/ml respectively. Statistical analysis showed no significant difference ($p > 0.05$) in the growth of the isolates at the various pH ranges.

The ability of the isolates to utilize various nitrogen sources was tested. The isolates were able to utilize all types of nitrogen sources tested containing ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$), sodium nitrate (NaNO_3) and ammonium chloride

(NH_4Cl). Ammonium chloride was the most utilized nitrogen source in this study with *Pseudomonas* sp producing the highest growth of 2.37×10^5 cfu/ml, followed closely by *Bacillus* sp with 2.17×10^5 cfu/ml while *Klebsiella* sp was 1.93×10^5 cfu/ml. Sodium nitrate was the second best utilized nitrogen source while ammonium sulphate was the least utilized with growth of 1.35×10^5 cfu/ml, 1.12×10^5 cfu/ml, and 1.08×10^5 cfu/ml for *Pseudomonas*, *Bacillus* and *Klebsiella* species respectively, statistical analysis at ($p < 0.05$) showed a significant difference between the tested nitrogen sources and the growth rate. Nitrogen sources play an important role in the production of biosurfactants, because bacteria require nitrogen to complete its metabolic pathways. (Banat et al., 2000).

Effects of Physical and Nutrient Parameters on the Utilization of Crude Oil

The effects of these isolates on the utilization of crude oil were tested. At the various pH ranges tested, percentage loss of oil was however not statistically significant ($p > 0.05$). At pH 6.5, about 76% loss of oil was recorded by *Pseudomonas* sp. *Bacillus* sp recorded a 68% loss at pH of 7.5 while *Klebsiella* sp had a 58% loss at pH of 7.0.

The ability of the isolates to utilize crude oil supplemented with different nitrogen sources were tested and no statistical difference ($p > 0.05$) was recorded. Percentage loss of 67%, 63% and 51.8% loss of crude oil were recorded for *Pseudomonas*, *Bacillus* and *Klebsiella* species respectively when supplemented with ammonium chloride. Ammonium chloride was the best utilized nitrogen source. Sodium nitrate recorded a 62.4%, 56.7% and 44.4% loss for *Pseudomonas*, *Bacillus* and *Klebsiella* species respectively. Ammonium sulphate was the least utilized with 53.2%, 46.7%, and 36% loss of crude oil for *Pseudomonas*, *Bacillus* and *Klebsiella* species respectively.

Crude Oil Utilization Studies

The use of microorganisms to degrade crude oil is not uncommon since the first publication of bacterial growth on petroleum hydrocarbons (Atlas, 1981, Gerson, 1985, Hidebrandt and Wilson, 1991). Microorganisms of soil and marine habitat are the major sources of bacteria responsible for utilizing petroleum hydrocarbons (Bossert and Bartha, 1984, Antai and Mgbomo, 1989). In this study, *Pseudomonas*, *Bacillus*, and *Klebsiella* species were used for the utilization studies. After 28 days of incubation, it was observed that all the isolates utilized crude oil at different degrees. The rate of utilization was minimum on day zero and subsequently increased with increase in the days of incubation. The highest oil utilization was observed in *Pseudomonas* sp (77.5%), this was followed by *Bacillus* sp (66.8%) and *Klebsiella* sp (54.2%). Genus *Pseudomonas* has been implicated by many investigators to be a potent hydrocarbon utilizer (Udotong, 1995, Essien et al., 1997 and Ezeji et al., 2005). The high degrading ability of *Pseudomonas* sp recorded in this study is however not uncommon and is consistent with other reports. Many studies have also reported the efficiency of *Pseudomonas* sp in degradation of hydrocarbon such as benzenes (Munoz et al., 2007), toluene, P-xylene (Yu et al., 2001), biphenyl (Ohta et al., 2001).

Bacillus sp capable of degrading hydrocarbons have also been reported (Nwaogu et al., 2008). Linda, et al., (2016) and German, et al., (2016) reported the degradation ability of *Klebsiella* sp. The degrading high ability of *Pseudomonas* sp in this study follows the same trend with the reports of Das and Mukherjee, (2007), Ezeji et al., (2005). However there was a significant difference in the weight of crude oil degraded at ($p < 0.05$).

The degradation of crude oil by microorganisms could be said to be a natural process by which the majority of the pollutant (oil) are being used as organic carbon sources, which results to the breakdown of petroleum compounds, transformation into other organic compounds or as an energy source and or production of other biological product. (Eniola et al., 2014). This isolates were able to degrade and utilize crude oil because microorganisms have been reported to be equipped with enzyme systems which enables them to utilize and degrade petroleum hydrocarbons as source of carbon and energy (Antai and Mgbomo, 1993, Ezeji et al., 2005)

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