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Biodegradation of Hydrocarbons in Untreated Produced Water (from an Oil field in the Warri, Delta State, Nigeria) Using Indigenous Bacterial Cultures.

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Abstract
Produced water is the largest volume of by-product generated during oil and gas recovery operations. Its contact with crude oil during extraction, results to the presence of high hydrocarbon content in it. Improper disposal of this produced water to the environment could cause hazardous effects. The objectives of this research are; to determine the mix bacteria population present in the produced water sample, to characterize, identify and isolate the hydrocarbon degrading bacteria found in the sample, and to cultivate a pure bacteria culture to degrade the hydrocarbon content of the sample. Five (5) hydrocarbon degrading bacteria such as: E. Coli, MicrococusLetus, Staphylococcus Aureus, BasillusSubstilis and Pseudomonas Aeruginosa were identified in the mix bacteria population, the hydrocarbon degrading bacteria with the highest population (which is Staphylococcus Aureus) was used for the pure bacteria culture using oil agar medium method. After its cultivation, a concentration of 10^8*cell per ml was used as a bio-stimulant to degrade the hydrocarbons in the sample. The initial total petroleum hydrocarbon content calculated in the sample was 125.513mg/l, after its incubation period of 15 days using the pure bacteria culture, the value of the hydrocarbon contents was then calculated and found to be 84.527mg/l. Thus, the hydrocarbon content removal efficiency in the sample of 425ml volume was calculated to be 32.655%. It is therefore recommended that for more efficient result, a mix hydrocarbon degrading bacteria culture of the listed hydrocarbon degrading bacteria should be used to carry out the experiment, which can produce 98% biodegradation according to the other researchers. In conclusion, the finding provides knowledge of hydrocarbon degradation bacteria present in the sample.*
Keywords*: Produced Water, Biodegradation, Hydrocarbon Content, Pure Bacteria Culture, Removal Efficiency*

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I. Introduction

The Oil & Gas industry requires water for extracting of oil from underground sources as well as refining crude oil (petroleum). Natural water or formation water is always found together with petroleum in reservoirs. This present water is slightly acidic and sits below the hydrocarbons in porous reservoir media. During the extraction of oil and gas, there exists a reduction in reservoir pressure, and additional water is usually injected into the reservoir water layer to maintain hydraulic pressure and enhance oil recovery [1]. In addition to injected water, there can be water breakthrough from outside the reservoir area, and as oil and gas production continues, the time comes when formation water reaches production well, and production of water begins alongside the hydrocarbons. This hydrocarbon contaminated water is known as produced water, accounting for the largest volume of by-product generated during oil and gas recovery operations. It is a mixture of injected water, formation water, hydrocarbons and chemicals added during the production and treatment processes [2].

Produced water contains hazardous pollutants of both organic and inorganic materials, thus should not be discharged directly to the environment. Uncontrolled discharge can lead to environmental damage, adversely affecting the lives of animals and plants and the quality of groundwater.

Produced water is the largest volume waste stream in the crude oil exploration and production process. Considering the global production of barrels of oil and related water, water to oil ratio is approximately 3:1 [3]. Global volume of produce water generated is up to 39.5 Mm3 /day, although the water to oil ratio is increasing. However, by 2025, due to the ageing wells, the water to oil ratio is expected to reach an average of 12 (v/v) for onshore crude oil resources. This will reinforce the growth of the market of produced water's management [3].

Bioremediation is one of the forms of biodegradation which involves the use of microorganisms to detoxify or remove organic and inorganic xenobiotic compounds from the environment. The process relies upon microbial enzymatic activities to degrade the contaminants from the environment [4]. Numerous microorganisms can utilize oil as source of food, and many of them produce potent surface-active compounds that can emulsify oil in water and facilitate its removal. Indeed, many studies have revealed that there is a large number of hydrocarbon-degrading bacteria in oil-rich environments, such as oil spill areas and oil reservoirs [5], and that their abundance and quantity are closely related to the types of petroleum hydrocarbons and the surrounding environmental factors [6]. Many normal and extreme bacterial species have been isolated and utilized as biodegraders for dealing with petroleum hydrocarbons. The degradation pathways of a variety of petroleum hydrocarbons (e.g., aliphatics and polyaromatics) have been shown to employ oxidizing reactions; however, these pathways differ greatly because of the specific oxygenases found in different bacterial species.

During initiation of biodegradation, the hydrocarbon must first be functionalized and currently it has been recognized that microbes have evolved an astonishing diverse range of activation (functionalizing) reactions [7]. These hydrocarbon activation mechanisms are different in aerobic and anaerobic micro- organisms [8]. Under aerobic conditions, oxygen is used as a co-substrate in both mono and dioxygenase reactions that facilitate the terminal or sub-terminal hydroxylation of alkanes as well as the mono- and di- hydroxylation of the aromatic hydrocarbons [9]. Under anaerobic conditions, some proposed reactions comprise; methylation of unsubstituted aromatics, addition of fumarate by glycyl-radical enzymes, water-mediated hydroxylation using molybdenum bound enzymes of an alkyl substituent via dehydrogenase as well as carboxylation catalyzed by uncharacterized enzymes which may represent a combination of the methylation reaction followed by the fumarate addition reaction [9].

The scope of this study is aimed at Identification, Isolation and Characterization of hydrocarbon degrading bacterial in oil field produced water from oil and gas exploration industries (Warri exploration site). Assessing and evaluating the pollution potential of the sampled produced water and also treatment to ensure clean water for both the environment and general human use, aquatic and plant life without any negative effect to the environment, soil, aquatic life like fishes, plants etc. using isolated degrading bacteria.

II. Materials and Methods

2.1 Materials and Apparatus

The materials and apparatus used for this experiment include;

1. Produced water sample collected from Warri Exploration site 2. Rotary incubator shaker: a device used to glow and maintain bacteria or fungi cultures 3. 250ml separating glass funnels 4. 10ml pipette 5. UV-Visible Spectrophotometer: used to measure the concentration of solutes in a solution by measuring the amount of light that's absorbed by the solution 6. Cuvette 7. Conical flask and beaker 8. Microscope

2.2 Collection of Sample

Produced water sample needed for the experiment was collected from Warri exploration site and stored in a sterilized 5L (Five Litres) container.

2.3 Bacteria Culture Preparation

For the purpose of this research work a combination of Mineral Salt Medium (MSM) of Zajic and Supplisson [10] and oil agar (OA) was used for the isolation of hydrocarbon degrading bacteria from the produced water sample and the pure bacteria culture was grown at room temperature (300C).

2.4 Isolation of Pure Bacteria Culture

Bushnell Haas (BH) [11] agar/broth medium was used for the isolation of hydrocarbon degrading bacteria present in the produced water sample. This Mineral Salt Medium (MSM) contains all nutrients except the carbon source.

2.5 Characterization and Identification of Bacteria Isolates

Characterization of the isolated bacteria culture was done with the methods which include; the morphological, microscopic and biochemical method. Identification of the isolated bacteria from produced water sample after characterization was achieved by comparing their experimental characteristics with those of known taxa (class) as described by Buchanan and Gibbons [12] in Bergey‟s Manual of Determinative Bacteriology.

2.6 Biochemical Tests

Biochemical tests performed on each of the bacteria isolates according to standard methods [13] include the under listed tests:

2.6.1 Catalase Test; Three drops of hydrogen peroxide (3%) were added into the overnight grown culture in the test tubes formation of vigorous bubbles indicate positive catalase test while no or very few bubbles produced indicate negative catalase test.

2.6.2 Casein Hydrolysis; Isolates were grown overnight in nutrient broth and inoculated on to a Skim Milk Agar and incubated at 30 °C for 48h. A clear zone was formed around the isolates against the white background, which indicate the casein hydrolysis activity of the isolates.

2.6.3 Urease Test**;** The pure bacterial isolates were inoculated into urea broth and incubated at 30°C for 24hrs, the colour changes from yellow to pink which indicate that there was a positive test for urease production.

2.6.4 Starch (glucose) Test; Isolates were grown overnight in nutrient broth and inoculated into starch agar medium and incubated at 30 °C for 48h. The plates were flooded with Gram Iodine. Clear area was formed around the isolate against the blue-black background, which indicates starch hydrolysis.

2.6.7 Indole Test; A sterilized test tubes containing 4mL of tryptophan broth, was inoculated aseptically by taking the growth for 24hrs culture. The tube was incubated at 370C for 24hrs and 0.5mL of Kovac‟s reagent was added to the broth culture. Formation of a pink to red colour (cherry-red ring) in the reagent on top of medium within seconds of adding the reagent, indicate a positive indole test while no colour change even after the addition of the reagent indicate indole negative test.

2.6.8 Oxidase Test; Three drops of freshly prepared oxidase reagent were added to piece of filter paper placed in a clean petri dish. A colony of the bacteria isolate was removed using a glass rod and was smeared on the filter paper. The development of a blue-purple colour within 10 seconds was considered as a positive oxidase test [13].

2.6.9 Motility Test; A straight needle was used to touch a colony of a young 24hrs culture growing on agar medium. And it was stabbed once to a depth of only 1/2 inch in the middle of the tube. The needle was kept in the same line it entered as it was removed from the medium. It was incubated at 370C and examine daily for up to 7days. Diffuse, hazy growths that spread 45 through the medium rendering it slightly opaque indicate a positive motility test while growth that is confined to the stab-line, with sharply defined margins and leaving the surrounding medium clearly transparent indicate a negative test.

2.6.10 Methyl Red (MR) Test; Two tubes containing MR Broth with a pure culture of the microorganisms under investigation was inoculated. The tubes were incubated at 350C for 48hours and 5 drops of the methyl red indicator solution was added to the first tube. A positive reaction is indicated if the colour of the medium changes to red within a few minutes.

2.6.11 Citrate utilization Test;this test is based on the ability of an organism to use citrate as its only source of carbon and ammonia as its only source of nitrogen. A loopful of the bacteria isolate culture was transferred into a test tube containing 4mL of modified koser‟s citrate medium (+ bromothymol blue). The tubes were incubated at room temperature (300C) for up to 4 days checking daily for growth. A blue colour of the medium was considered as positive test of citrate utilization as only source of carbon [13].

2.6.12 Coagulase Test; Dilution of the plasma in saline (0.85% NaCl) was prepared and placed in 1ml of diluted rabbit plasma to give a milky suspension. The test tube was incubated at 350C in a water bath for 4 hours. It was examined at 1, 2, and 4 hours for clot formation by tilting the tube through 900. Formation of clot of any size indicates a positive coagulase test. In the case of no clot (negative coagulase test), tubes were left overnight at room temperature and re-examine.

2.7 Growth and Hydrocarbon Removal

Each of the selected bacteria strain (1.0 Ml of 0.8 OD bacteria suspension) was grown on 100mL BH medium supplemented with 1% (v/v) hydrocarbon for 7 days at 300C, on rotator shaker (150rpm). The growth of isolates was daily assessed by measuring the Optical Density (OD) at λ=480nm. After the period of incubation, hydrocarbon was extracted from medium using 50mL of cyclohexane and, after the organic solvent volatilization, the residual hydrocarbon was evaluated gravimetrically.

2.8 Estimation of Hydrocarbon Biodegradation Efficiency by Gravimetric Analysis

The isolates amounted to cells/mL and were inoculated into 100 mL BH MSM supplemented with 5% hydrocarbon dispensed in 250 mL conical flasks on a rotary shaker (150 rpm), and incubated at 30 °C for 10 and 15 days. The residual concentration of hydrocarbon was assessed using the method with slight modification. Thus, 1% HCl was added into the culture to stop the bacterial activity and the residual were extracted from the whole content using petroleum ether and acetone (1:1 v/v ratio). Then the flask was placed on the shaker at 120 rpm for 20 minutes and oil-containing solvent was collected from the upper portion of the flask and poured into the pre-weighted petri-plate. It was repeated three times to ensure complete extraction. The extracted component was allowed to evaporate in a hot air oven at 72 °C. Then, the residual hydrocarbon was calculated as percentage of degradation using the following formula

$$\text{Percentage Degradation (\%)} = \frac{\text{Initial Concentration of Hydrocarbon} - \text{Final Concentration of Hyrdocarbon}}{\text{Initial Concentration of Hydrocarbon}} \text{ x } 100$$

III. Results and Discussion

3.1 Isolation, Characterization and Identification of Bacteria isolates

The cultural and morphological characterization resulted in the suspicion of E. coli, Micrococcus Letus, Staphylococcus Aureus, Basillussubstilis and Pseudomonas aeruginosa was made. Results of these experiments are as shown in Table 1.

Table 1: Cultural andMorphologicalCharacteristics

Isolates	Shape	Margin	Elevation	Size	Colour	Colony surface	Gram reaction	Cell type	Cell arrangement	Suspected bacteria
A	Round	Rough	Flat	1mm	Green	Moist	-ve	Rod	Singly	Pseudomonas aeruginosa
B	Round	Smooth	Raised	1mm	Golden yellow	Moist	+ve	Cocci	Cluster	Staphylococcus aureus
C	Round	Smooth	Raised	0.5mm	Pink	Moist	-ve	Rod	Chain	E. coli
D	Round	Rough	Raised	0.5mm	Yellow	Dry	+ve	Cocci	Cluster	Micrococcus Letus
F	Round	Smooth	Raised	1mm	White	Dry	+ve	Cocci	Cluster	Staphylococcus Epididymis

The biochemical characterization of hydrocarbon degrading bacteria further confirmed the presence of B. substilis, M. letus, E. coli, and P. aeruginosa in the hydrocarbon contaminated water samples. Result of this experiment is as shown in Table 2

Table 2: Biochemical Characteristics

Isolates	Catalase	Coagulase	Oxidase	Urease	Glucose	Citrate	Motility	MR	Spore	Indole	Suspected Bacteria
A	-ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve	-ve	Pseudomonas aeruginosa
B	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	Staphylococcus Aureus
C	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	E. coli
D	+ve	-ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	Micrococcus Letus
F	+ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	Staphylococcus Epididymis

Table 3 represent a summary of identification of five (5) bacterial isolates, which have been suspected from cultural and morphological characteristics and biochemical characteristic, the results compare with Bergey‟s Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974)

Table3: Identification of Bacteria Isolate

ISOLATES	IDENTIFICATION
A	Pseudomonas aeruginosa
B	Staphylococcus Aureus
C	Escherichia coli
D	Micrococcus Letus
F	Staphylococcus Epididymis

3.2 Nitrate-nitrogen (NO3-N) content

There was a reduction in the concentration of NO3-N in the contaminated PAHs polluted water suggesting that the process of nitrification might have reduced crude oil contamination. Hydrocarbon- utilizing microbes such as Azobacterspp normally become more abundant while nitrifying bacteria such as Nitrosomonasspp become reduced in number. This probably explains the relatively lower values of NO3-N obtained after degradation.

3.3 Calibration of UV-Visible Spectrophotometer in Concentration
The UV-Visible spectrophotometer records the concentration of hydrocarbon content of the produced water in absorbance (nm). This absorbance in terms of concentration is shown in table 4. The plotting of the values in a straight line helps to get the concentration of any absorbance through the equation of a straight line in Figure 1

Table4:CalibrationTable

TPH (g/200ml)	Absorbance @ 480nm
0.016	0.157
0.030	0.389
0.055	0.786

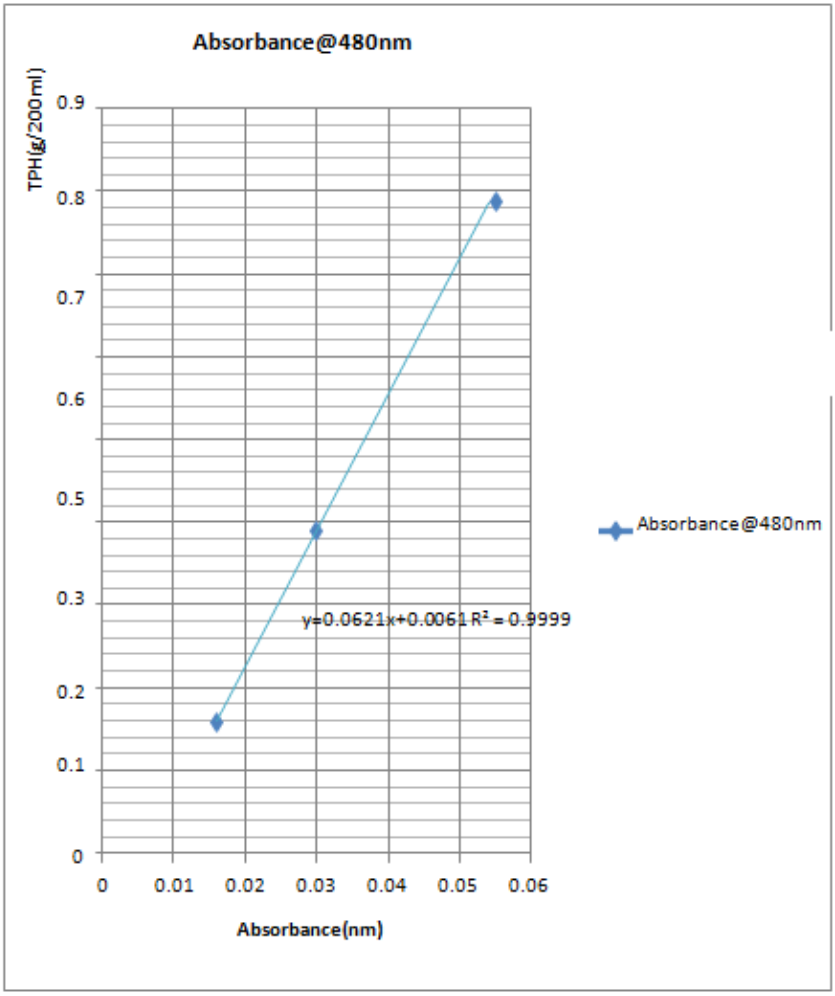

Figure1:Calibration Curve

3.4 Hydrocarbon Biodegradation of produced water
The result of the concentration of hydrocarbon present in the produced water measured at 3 days interval using spectrophotometer is show in the table 5 below.

Table 5: Biodegradation of hydrocarbon in produced water concentration

Day	Date	Absorbance (nm)	TPH (g/200ml)	TPH (mg/L)
1	18/6/2021	0.306	0.025103	125.513
2	21/6/2021	0.247	0.021439	107.194
3	24/6/2021	0.203	0.018706	93.532

4	27/6/2021	0.182	0.017402	87.011
5	30/6/2021	0.178	0.017154	85.769
6	03/7/2021	0.174	0.016905	84.527

3.5 Biodegradation efficiency

The efficiency of the biodegradation showed that the rate of degradation of the hydrocarbon increased with time (table 6) and the biodegradation curve decreased (Figure 2)

Table 6: biodegradation efficiency

Day	TPH (mg/L)	Biodegradation efficiency (%)
1	125.513	-
2	107.194	14.595
3	93.532	25.48
4	87.011	30.676
5	85.769	31.665
6	84.527	32.655

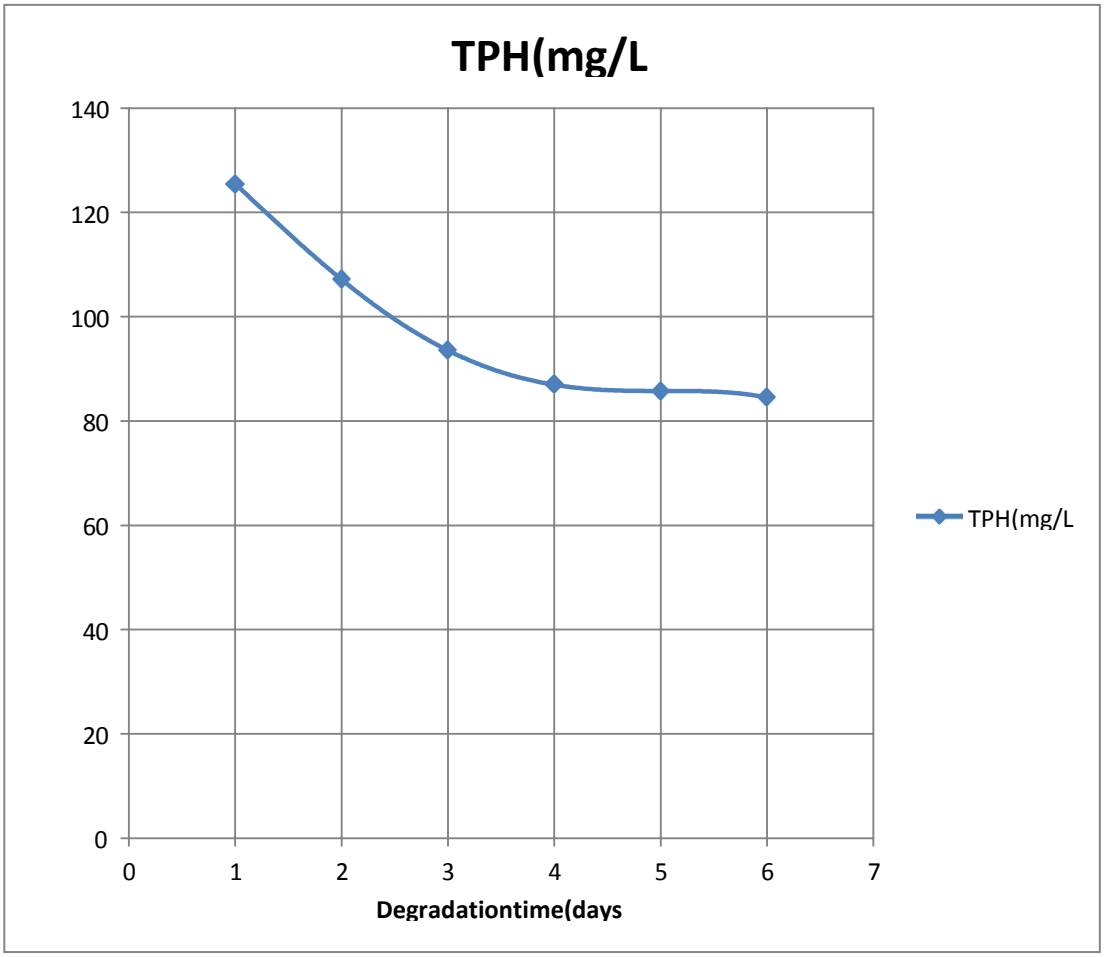

Figure 2: biodegradation curve

3.6 Biodegradation efficiency

After the incubation period of 15 days the biodegradation bacteria could only degrade about 32.655% of the total hydrocarbon content.

IV. Conclusion

This study carried out using pure single strains (i.e. bio-stimulation method of bioremediation) has an efficiency of about 33%, other studies by group of researchers have shown a consortium of several bacteria strains (i.e. bio-augmentation method of bioremediation) is required for complete mineralization of hydrocarbon contaminants given the complexity of hydrocarbon Petroleum products. Sathishkumer et al. [14] reported that biodegradation caused by mixture cultures is more effective than that caused by pure cultures of individual strains. Mukred et al. [15] found that a mixed bacteria consortium gives maximum of 98% degradation after 15 days.

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Competing Interests

Authors declare that no competing interests exist.

References

[1]. Igunnu, E.T., Chen, G.Z., 2012. Produced water treatment technologies. Int. J. Low-Carbon Technol. 1e23.
[2]. Strømgren, T., Sørstrøm, S.E., Schou, L., Kaarstad, I., Aunaas, T., Brakstad, O.G.,Johansen, Ø. 1995. Acute toxic effects of produced water in relation to chemical composition and dispersion. Mar. Environ. Res. 40, 147e169.
[3]. Arowoshola, L., Cope, G., David, V., Gasson, C., Gonzalez-mancho, C., Kelleher, M. Lang, H., Uzelac, J 2011. Produced water market. Opportunities in the Oil, Shale and Gas Sectors in North America. A Global Water Intelligence publication ISBN: 978-907467-14-1.
[4]. Philp, J. C., Whiteley A. S, Ciric Land Bailey, M. J. 2005. Monitoring bioremediation, in Bioremediation – Applied Microbial Solutions for Real-World Environmental Cleanup, ed by Atlas RM and Philp J. ASM Press, Washington, pp 237–268
[5]. Yang, Y., Wang, J., Liao, J., Xie, S., and Huang, Y. 2015. Abundance and diversity of soil petroleum hydrocarbon-degrading microbial communities in oil exploring areas. Appl. Microbiol. Biotechnol. 99, 1935–1946. doi: 10.1007/s00253-014-6074-z
[6]. Varjani S. J., Gnansounou E. 2017. Microbial dynamics in petroleum oilfields and their relationship with physiological properties of petroleum oil reservoirs. Bioresour. Technol. 245 1258–1265. 10.1016/j.biortech.2017.08.028
[7]. Isabel Natalia Sierra Garcia, Valeria Maia Oliveira 2013. Microbial Hydrocarbon Degradation: Efforts to Understand Biodegradation in Petroleum Reservoirs. DOI: 10.5772/55920
[8]. Widdel, F., Musat, F. 2010. Energetic and Other Quantitative Aspects of Microbial Hydrocarbon Utilization.Timmis, K.N. (eds) Handbook of Hydrocarbon and Lipid Microbiology. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-540-77587-4_57
[9]. Matthias Boll, Johann Heider 2010. Anaerobic Degradation of Hydrocarbons: Mechanisms of C–H-Bond Activation in the Absence of Oxygen. In book: Handbook of Hydrocarbon and Lipid Microbiology. DOI:10.1007/978-3-540-77587-4_71
[10]. Zajic, J.E. and Supplisson, B. 1972. Emulsification and degradation of "Bunker C" fuel oil by microorganisms.Biotechnol. Bioeng, 14: 331-343.https://doi.org/10.1002/bit.260140306
[11]. Bushell, LD, and Haas, H.F. 1940. The utilization of certain hydrocarbons by microorganisms. Journal of Bacteriology. 41:653-673.
[12]. LEVINE, N.D. (1975), Buchanan, R. E. & Gibbons, N. E., eds. 1974. Bergey's Manual of Determinative Bacteriology. The Journal of Protozoology, 22: 7-7. https://doi.org/10.1111/j.1550-7408.1975.tb00935.x
[13]. Cheesbrough, M. 1991. Medical Laboratory Manual for Tropical countries, Vol II, 1 ed. University Press, Cambridge.
[14]. Sathishkumar M., Arthur Raj Binupriya, Sang-HoBaik, Sei-Eok Yun 2008. Biodegradation of Crude Oil by Individual Bacterial Strains and a Mixed Bacterial Consortium Isolated from Hydrocarbon Contaminated Areas. CLEAN - Soil Air Water 36(1):92 – 96 DOI: 10.1002/clen.200700042
[15]. Abdualdaim Mohammed Mukred, Aidil Abdul Hamid, AinonHamzah, Wan Mohtar, Wan Yusoff 2008. Growth Enhancement of Effective Microorganisms for Bioremediation of Crude Oil Contaminated Waters. Pakistan Journal of Biological Sciences 11(13):1708-12. DOI: 10.3923/pjbs.2008.1708.1712