Isolation and Molecular Characterization of Acid Producing Bacteria from Oilfield Environments Located in the Niger Delta, Nigeria.

ABSTRACT

Acid producing bacteria are known and considered to be an important group of corrosive bacteria of that have economic importance in theto petroleum industry. In this research, acid producing bacteria were isolated from wasted water sample collected at from ten (10) oil field environments within theon Niger Delta Region. The multiple tube fermentation technique was used to isolate the bacteria. Phenol red dextrose broth was used as the microbiological medium tofor the isolation of the acid producing bacteria. The total heterotrophic bacteria count (THBC) was determined under aerobic and anaerobic condition using the standard plate count techniques. The boiling method was used to for the extraction of the acid producing bacterial DNA after growing in Luria Bertani broth. The extracted bacterial DNA were purified and quantified before PCR amplification. The PCR amplicons were subjected to gel electrophoresis. The bacterial DNA bands were quantified using the 1500bp ladder. The result obtained showed that some of the acid producing bacteria isolated could survive as facultative microorganisms belonging to genera such as *Klebsiella* sp., *Pantoea* sp., *Escherichia* sp., *Providentia* sp., *Proteus* sp., *Shewanella* sp., *Myroides* sp. and *Pseudomonas* sp.. There was growth in all samples under aerobic condition with a THBC ranging from $3.602x10^2$ Cfu/ml – $4.698 x10^2$ Cfu/ml while the range was within $3.301 x10^2$ Cfu/ml – $5.676 x10^2$ Cfu/ml under anaerobic condition. For the physicochemical parameters determined, the temperature range for all samples was within 23.9^o C – 24.8^o C; the pH was within 7.24 – 8.10; the total dissolved solids was within 470mg/ml – 16160mg/ml and the conductivity was within 1.885 µs/cm – 845.2 µs/cm. The results also showed that acid producing bacteria grow mostly under aerobic condition unlike the SRB.

Key words: produced water, acid producing bacteria, corrosive, molecular technique, facultative microorganisms

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Introduction

Produced water is the Iindustrial waste waterwastewater which is a by-product of hydrocarbon exploration and production. It is formed from sea water and hydrocarbon formation water [1,2]. Produced water contains organic and inorganic compounds. The compounds consist of dispersed oil components grease, heavy metals, radionuclides, microorganisms, scale products, dissolved oxygen, hydraulic fluid chemicals, salts, dissolved formation minerals and gases [3,4].

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Diverse physiological groups of microorganisms are present in produced water including those associated with the corrosion of oil and gas facilities. The acid producing bacteria (fermentative bacteria) produce organic acids which are corrosive and can serve as precursor metabolites for other corrosive bacteria which aid the corrosive activities of these bacteria. Some aerobic bacteria that make up the microbial community in an oilfield environment usually enter during drilling or the application of injection water for pressure build up [5]. The genome of aerobic hydrocarbon utilizing bacteria can be determined enzymatically [6].

Molecular techniques are currently applied in the study of microbial community structure and composition to obtain the true functional activity and phylogenetic diversity of metabolically active microbes in an oilfield environment [7-9]. The description of the microbial community of an environmental sample can be done using the ribosomal RNA to obtain the libraries of the cDNA of the 16S rRNA fragments [10,11]. The 16S rRNA clone libraries and sequences from the total microbial cell DNA can also be used to determine the microbial diversity in formation water from oil production wells [8,12-14]. The present study is, therefore, on the molecular characterization of acid producing bacteria from oilfield environments located in the Niger Delta, Nigeria.

Materials and Method

Waste WaterWastewater Sample Collection and Transport

Produced water samples were collected from injection wells (8) and flow stations (2) in oil field environments from Imo river, Umuechem, Cawthorn channel and Benisede located within the oil rich region of the Niger Delta, Nigeria. The samples were transported in sample bottles covered in black cellophane bag.

Physicochemical Analyses

The physicochemical parameters that were analysed in the produced water samples include: the tTemperature, pH, electrical conductivity and the total dissolved solids. The tTemperature and the pH werewere determined using the Thermo Scientific Orion Star A214 pH/ISE meter while the total dissolved solids and the electrical conductivity were determined using the YSI 3200 Conductivity Instrument [15].

Microbiological analyses

Estimation of total heterotrophic bacteria in the waste waterwastewater samples

The total heterotrophic bacterial population was determined under aerobic and anaerobic conditions using the standard plate count method of enumeration. 0.1ml dilutions of the waste waterwastewater samples were aseptically inoculated into sterile plates of standard plate count

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agar (SPCA). A sterile glass rod (hockey stick) was used to spread the inoculum in an even pattern on the surface of the agar plates in triplicates [16]. The cultured plates were incubated at 37^{o}C for 24 to 48 hours for the aerobic culture and for seven (7) to for the anaerobic culture.

The cultured plates of total viable counts were estimated as thus:

$$\text{CFU/ml} = \frac{\text{TVC X Dilution Factor}}{\text{Inoculum Volume}}$$

Isolation and Purification of Acid Producing Bacteria

The acid producing bacteria were isolated from produced water samples using Phenol red dextrose culture broth. The broth medium was prepared by mixing 10g of peptone, 5g of dextrose, 5g of sodium chloride and 18mg of phenol red powder with 1litre of distilled water. The medium was autoclaved at 121 ^{o} C for 15 minutes before use. The multiple tube fermentation technique was adopted for the bio-corrosion studies involving acid producing bacteria [16]. The inoculated broth was incubated at 37 ^{o} C for 7 days under aerobic and anaerobic condition during the study.
The isolates were purified by sub-culturing in MacConkey agar as a differential/ selective medium for isolation. The pure isolates were used for the molecular studies.

Extraction and Purification of Acid Producing Bacterial DNA

The boiling method was used for the extraction of the acid producing bacterial DNA. Pure colonies of acid producing bacteria were inoculated into 6 ml of Luria Bertani broth (LB) and incubated at 37^{o}C for 6-10 hours. The bacterial isolates in the LB broth was spun at 12000rpm for 3 min followed by the addition of 500 ul of normal saline to the Ependorff tube containing the cell DNA sample. The tTubes were heated at 95^{o}C for 20 min, then. The tubesi t were fast cooled on ice followed by spinning at 12000rpm for 3 min. The cell DNA supernatant was kept at -10^{o}C for further procedures. After that, Tthe extracted cell genomic DNA was quantitated by using the Nanodrop 1000 spectrophotometer.

16S rRNA Amplification and Sequencing

The amplification was done by using the 16s rRNA region of the rRNA gene of the isolates. The primers used for the amplification are 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5' CGGTTACCTTGTTACGACTT-3' on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 micro-litres for 35 cycles. The initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The amplicons were resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator.

Sequencing

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The BigDye Terminator Kit on a 3510 ABI Sequencer was used to perform the sequencing. The analysis was done by Inqaba Biotechnological, Pretoria, South Africa. The final volume of the sequencing was 10ul. 0.25ul BigDye@ terminator v1.1/v3.1, 2.25ul of 5x BigDye sequencing buffer, 10uM Primer, PCR Primer and 2-10ng PCR template per 100bp were used as the components for the sequencing and_ the optimum conditions are 32 cycles of 96^{o}C for 10s, 55^{o}C for 5s and 60 ^{o}C for 4min.

Phylogenetic Analysis

Bioinformatics algorithm Trace edit was used to edit the sequences obtained. BLASTN was electronically used to download similar sequences from the National Center for Biotechnology Information (NCBI) database. MAFFT was used to align the sequences. The Neighbor-Joining method in MEGA 6.0 was adopted to infer the evolutionary history of the isolates [17]. The bootstrap consensus tree predicted from 500_replicates [18] was taken to represent the evolutionary history of the taxa determined. The Jukes- Cantor method was used to compute the evolutionary distances [19].

Results

The result of the physicochemical parameters of the produced water is given in Figures 1-4 which show the graphicalgraphically view of the relationship of the values of each parameter with the sample source.

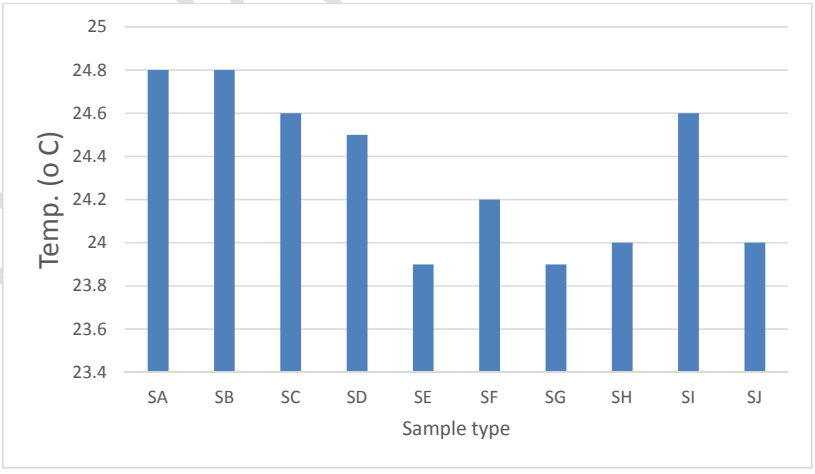

Figure 1: The temperature values of the produced water samples

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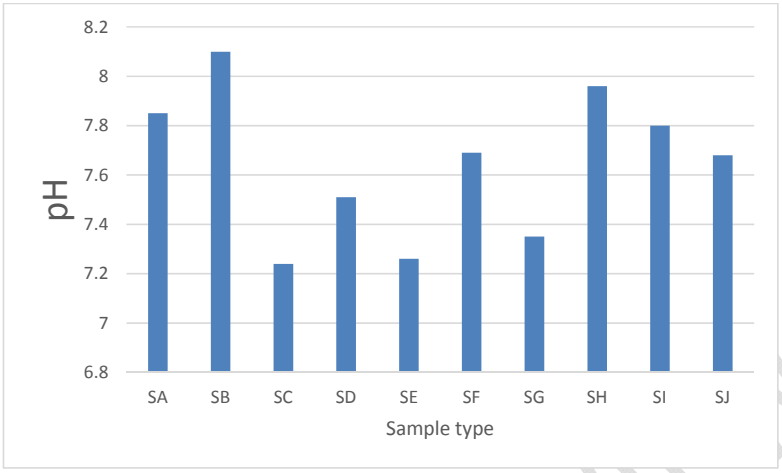

Figure 2: The pH values of the produced water samples

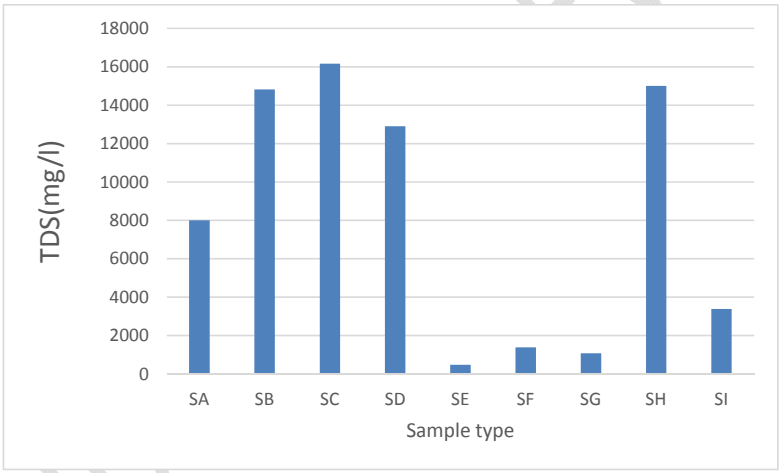

Figure 3: The values of Total Dissolved Solids

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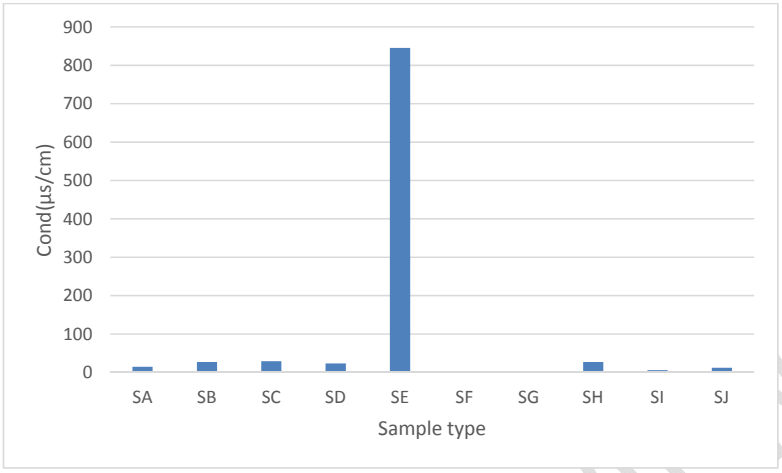

Figure 4: The values of the electrical conductivity

The microbiological analyses results **of the microbiological analyses** of **the** produced water sample is given in Figures 5 – 7.

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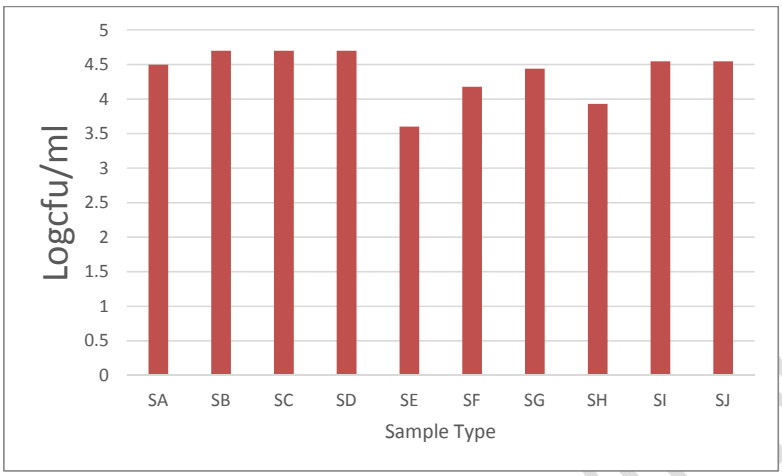

Figure 5: Total heterotrophic bacteria population (aerobic)

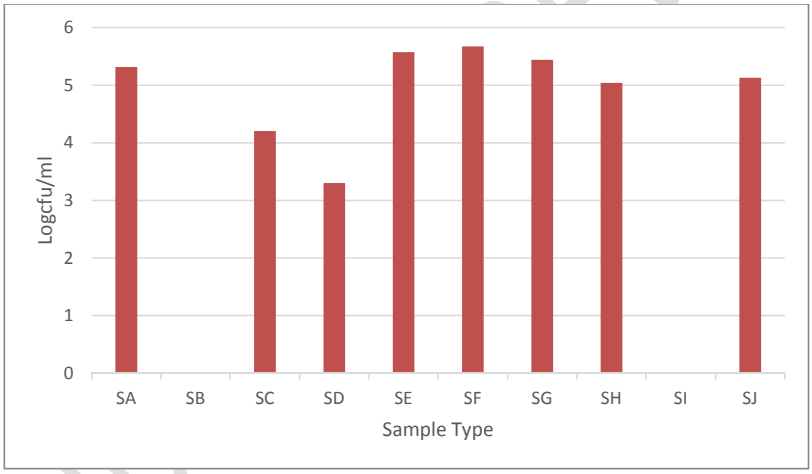

Figure 6: Total heterotrophic bacteria population (anaerobic)

Molecular Characterization of Acid Producing Bacteria from Produced Water

The result of the molecular identification of the corrosive bacteria in the produced water sample is given below:

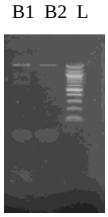

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Plate 1: Agarose gel electrophoresis of the 16S rRNA gene of the study bacterial isolates. Lanes B1 and B2 represent the 16SrRNA gene bands (1500bp), lane L represents the 100bp molecular ladder.

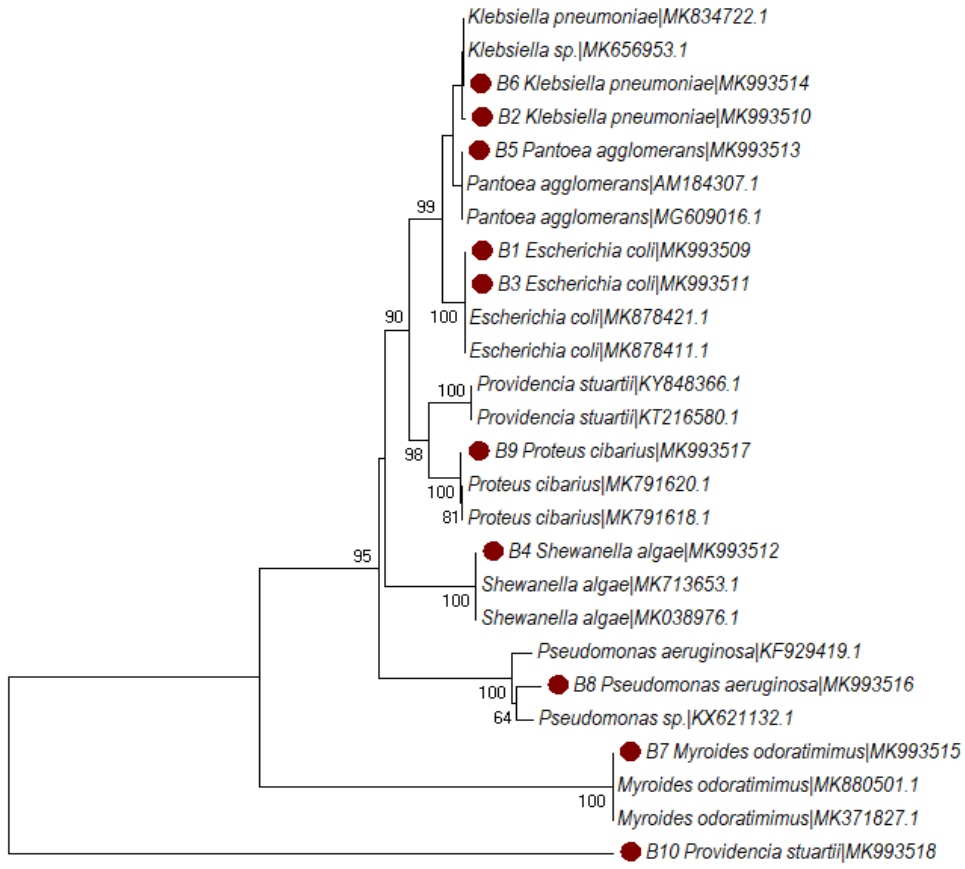

Figure 7: Phylogenetic Tree of Acid Producing Bacteria from Produced Water.

Discussion

Temperature

The temperature values were highest for the flow station samples (SA & SB), while that for the injection wells (SC-SJ) were all within similar ranges (23.4 - 24.6^{o}C). The temperatures for all the samples were slightly below ambient temperature. This indicates influence thatof environmental temperature on the water environment where the corrosive bacteria can be found as temperature changes can occur due to atmospheric conditions and seasonal variations. Awoyemi *et al*. [20] reported a temperature range of 26.10 to 26.55^{o}C for rainy season and 28.10 ^{o}C for dry season for both groundwater and surface water. This report clearly indicates that the environment greatly influenceinfluences the changes in temperature. Onojake *et al*. [21] also reported thate temperature of produced water from oilfield location to be within the a range of 21.9 to 24.7^{o}C.

pH

The pH values for all the samples were within slightly the same slightly alkaline range (7.24-8.10). Corrosive bacteria can survive such pH that is not extreme, although they would survive best under acidic condition. The pH values were within the permissible to the limit of pH (7.47 to 8.50) for inland and near shore reported by Onojake *et al.* [21].

Total Dissolved Solids (TDS)

The TDS indicate the presence of dissolved heavy metal ions and salts in produced water [21]. The TDS values were high for SB, SC SD and SH within a the range of (470mg/l - 16160mg/l, indicating greater degree of pollution by the dissolved substances presence of dissolved substances in the samples. The values of 80% of the samples were above the regulatory limit of 2000mg/ml for inland area by WHO [21]. Only three (3) samples had TDS values within 400 to 1400mg/ml. Onojake *et al.* [21] reported TDS values for produced water ranging from 3200 to 7000mg/ml. TDS values also indicate greater microbial population in most of the produced water sample.

Electrical Conductivity

Electrical conductivity values show the purity level of purity of the produced water samples. The conductivity value was high for only SE (845.2µs/cm). The values for SF (2.425µs/cm) and SG (1.885µs/cm) were very negligible in comparison to all other sample values. Onojake *et al*. [21], reported conductivity values ranging from 126.50 to 198.00 µs/cm. The conductivity indicates the presence of dissolved salts and elements in the produced water samples. It is used to test the urity level of purity of water. The higher the conductivity, the lower the purity, the higher the microbial population degree of microbial population and MIC possibility of MIC, and the pollution rate of the produced water sample.

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Acid producing bacteria also known as fermentative bacteria can grow as facultative microorganisms. When grown under both aerobic and anaerobic conditions, it was observed that there was growth from all samples under aerobic condition within a short during than **the** anaerobic condition which took longer growth time. From **the** this study result, it can be inferred **that** that **the** acid producing bacteria **can** could survive in different environment and under different growth condition. This could be **can be seen as** a mode of ecological adaptation for survival strategy in certain environment. Microbial control specialists report [22] revealed that among **the** acid producing bacteria isolated from tank water and pipeline, *Shewanella* sp is associated with metal corrosion while *Klebsiella* sp is known for biofilm formation.

Among the microorganism's genera **of microorganisms** identified as acid producing bacteria that is **the** *Escherichia coli*, which for long was known to be **the** major faecal coliform bacteria of public health concern. Its occurrence in an oilfield environment is very strange but is a possibility in terms of species diversity, migration and species distribution in **the** environment based on **the** ability to adapt with ecological changes in different environment. It could also mean that **the** microorganism is a unique strain of *Escherichia coli* which possesses the mechanism or metabolic capacity to survive in a different environment. This school of thought also holds way for *Klebsiella* sp, *Providentia* sp and *Proteus* sp which are also among the group of coliform bacteria of public health importance due to their presence in groundwater [16]. In all, the presence of these group of bacteria in **the** produced water sample also indicates that there is obvious similarity in **the** environment where these bacteria can be found and isolated. Acid producing bacteria like other corrosive bacteria release metabolic products which **are seen as**are metabolic markers such as exo-enzymes linked with extracellular polymeric substances (EPS), organic and inorganic acids, nitrites, ammonia and sulphides. At some time, they can lead to **the** solid corrosion products formation **of solid corrosion products** [23,24]. *Pseudomonas* is an example of acid producing bacteria which releases organic acids which act as very aggressive metabolites that can lead to localized bio-deposit and cause pitting corrosion in pipeline which can spread to **the** entire surface of **the** metal structure. These bio-deposits act as traps and food for other corrosive microorganisms which lead to **the** formation of a complex matrix of bacterial biofilm that further set up a corrosion potential between **the** metal surface and **the** layer beneath **the** biofilm. Apart from being corrosive, as part of their benefit to **the** environment where they function, the acid producing bacteria because of their fermenting property can promote oil production by modifying the reservoir fluid and rock properties (cause rock mineralization). When added to reservoirs their bio-products can **effect and** improve oil production [25]. More so, Biji *et al*. [26] reported that microorganisms can synthesize useful products by fermenting cheap raw materials applicable in enhanced oil recovery. This makes microbial enhanced oil recovery to be very sustainable compared to chemical enhanced oil recovery because of **the** high cost of chemicals. It is also very interesting to note that **the** microbial products from **the** APB are biodegradable and environmentally friendly [26].

Conclusion

The presented study **on the**about isolation and characterization of acid producing bacteria has revealed the possibility of **the** coliform bacteria to be among **the** corrosive bacteria such as **the**

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sulphate reducing bacteria (SRB), iron oxidizing bacteria (IOB) , manganese oxidizing bacteria (MOB) .e.tc. involved in the biocorrosion of metals and industrial metallic materials. These corrosive bacteria have been and is still problematic to the durability and integrity of industrial facilities today.

References

1. Igunnu ET, Chen GZ. Produced water treatment technologies, *International Journal of Low-Carbon Technologies*. 2014; 9 (3): 157–177.

2. Reynolds RR. Produced water and associated issues: A manual for the independent operator, Oklahoma Geological Survey Open-File Report. 2003; 6:1–56.

3. Fakhru'l-Razi, A., A. Pendashteh, L. C. Abdullah, D. R. A. Biak, S. S. Madaeni, and Z. Z. Abidin (2009) Review of technologies for oil and gas produced water treatment, *Journal of Hazardous Materials*,170 (2-3) : 530 – 551.

4. Hansen BR, Davies SRH. Review of potential technologies for the removal of dissolved components from produced water. *Chemical Engineering Research and Design*. 1994; 72: 176–188.

5. An D, Caffrey SM., Soh J, Agrawal A, Brown D, *et al*. (2013). Metagenomics of hydrocarbon resource environments indicates aerobic taxa and genes to be unexpectedly common. *Environ. Sci. Technol*.47, 10708–10717.

6. Head IM, Gray ND, Larter SR. Life in the slow lane; biogeochemistry of biodegraded petroleum containing reservoirs and implications for energy recovery and carbon management. *Front. Microbiol*. 2014; 5:566.

7. Nazina TN, Grigor'yan AA, Shestakova NM, Babich TL, Ivoilov VS, Feng Q. Microbiological investigations of high-temperature horizons of the Kongdian petroleum reservoir in connection with field trial of a biotechnology for enhancement of oil recovery. *Microbiology*. 2007; 76: 287 – 296.

8. Bødtker G, Lysnes K, Torsvik T, Bjørnestad E Ø, Sunde E. Microbial analysis of back-flowed injection water from a nitrate treated North Sea oil reservoir. *J. Ind. Microbiol. Biotechnol*. 2009; 36, 439–450.

9. Gao PK, Li GQ, Tian HM, Wang YS, Sun HW, Ma T. Differences in microbial community composition between injection and production water samples of water flooding petroleum reservoirs. *Biogeosciences*. 2015; 12: 3403–3414.

10. Mills HJ, Martinez RJ, Story S, Sobecky PA. Characterization of microbial community structure in Gulf of Mexico gas hydrates: comparative analysis of DNA-and RNA-derived clone libraries. *Appl.Environ.Microbiol*. 2005; 71: 3235 – 3247.

UNDER PEER

11. Moeseneder MM, Arrieta JM, Herndl GH. A comparison of DNA- and RNA-based clone libraries from the same marine bacterio-plankton community. FEMS *Microbiol. Ecol*. 2005; 51: 341 – 352.

12. Duncan KE, Gieg LM, Parisi VA, Tanner RS, Suflita JM, Tringe GS. (). Biocorrosive thermophilic microbial communities in Alaskan North Slope oil facilities. *Environ. Sci. Technol*. 2009; 43:7977–7984.

13. Gieg LM, Davidova IA, Duncan KE, Suflita JM. Methanogenesis, sulfate reduction and crude oil biodegradation in hot Alaskan oilfields. *Environ. Microbiol*. 2010; 12: 3074–3086.

14. Van der Kraan GM, Bruining J, Lomans BP, Van Loosdrecht MCM, Muyzer G. Microbial diversity of an oil-water processing site and its associated oilfield: the possible role of microorganisms as information carriers from oil-associated environments. *FEMS Microbiol. Ecol*. 2010; 71: 428–443.

15. APHA. Standard Methods for the Examination of Water and Waste Water, 22nd Edition, American Public Health Association, Washington, D.C. 2012.

16. Briggs WF, Agwa OK, Abu GO. (). Quantitative Microbial Risk Assessment (QMRA) of Groundwater in Abonnema Community in Kalabari Kingdom, Rivers State, Nigeria. *Adv Biotech & Micro*. 2018; 8 (3) : 555740.

17. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution.* 1987; 4:406-425.

18. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution.* 1985; 39:783-791.

19. Jukes TH, Cantor CR. Evolution of protein molecules. In Munro HN, editor, *Mammalian Protein Metabolism*, pp. 21-132, Academic Press, New York. 1969.

20. Awoyemi OM, Achudume AC, Okoya AA. The Physicochemical Quality of Groundwater in Relation to Surface Water Pollution in Majidun Area of Ikorodu, Lagos State, Nigeria. *American Journal of Water Resources*. 2014; *2*(5), 126-133.

21. Onojake MC, Abanum UI. Evaluation and management of produced water from selected oil fields in Niger Delta, Nigeria. *Archives of Applied Science Research*, 2012; 4 (1):39-47.

22. Microbial Control Specialists Report (2015). Bacterial Diversity Analysis Syrinx Well Services, Project ID: OG142112

23. Beech IB, Gaylarde CC. (). Recent advances in the study of biocorrosion: an overview. Rev. *Microbiol*. 1999; 30 (3):117–190.

24. Beech IB, Sunner J. Biocorrosion: towards understanding interactions between biofilms and metals. *Curr. Opin. Biotechnol*. 2004; 15(3):181– 186.

UNDER PEER REVIEW

353 25. Patel J, Subrata B, Mayank K, Vivek R. Recent developments in microbial enhanced oil
354 recovery. *Renewable and Sustainable Energy Reviews*. 2015; 52:1539 – 1558.

355 26. Biji S, Saif NA, Yahya MA, Abdulkader EE, Ali SA, Sanket J. Microbial Enhanced Heavy
356 Oil Recovery by the Aid of Inhabitant Spore-Forming Bacteria: An Insight Review. 2014**.**

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