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Isolation and Molecular Characterization of Acid Producing Bacteria from Selected Oilfield Environments within the Niger Delta

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Authors' contributions

This work was carried out in collaboration among all authors. Author WFB designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors HOS and GCO managed the analyses of the study. Authors OMI and CJU managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Acid producing bacteria are considered an important group of corrosive bacteria that have economic importance to petroleum industry. In this research, acid producing bacteria were isolated from produced water sample collected at ten (10) oil field environments within the Niger Delta region. The multiple tube fermentation technique was used to isolate the bacteria while Phenol red dextrose broth was used as the microbiological medium for the isolation of the acid producing bacteria. Also total heterotrophic bacteria count (THBC) was determined under aerobic and anaerobic condition using the standard plate count technique and the boiling method used for the extraction of 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 1500bp ladder. The result obtained

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showed that some acid producing bacteria isolated could survive as facultative microorganisms belonging to genera such as *Klebsiella, Pantoea, Escherichia, Providentia, Proteus, Shewanella, Myroides and Pseudomonas.* There was growth in all samples under aerobic condition with a THBC ranging from $3.602x10^2$Cfu/ml – $4.698x10^2$Cfu/ml while the range was within $3.301x10^2$Cfu/ml – $5.676x10^2$Cfu/ml under anaerobic condition. For physicochemical parameters determined, temperature range for all samples was within 23.9ºC – 24.8ºC; pH was within 7.24 – 8.10; total dissolved solids was within 470 mg/ml – 16160 mg/ml and 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 sulphate reducing bacteria.

Keywords: Acid producing bacteria; corrosive; molecular technique; Niger Delta.

1. INTRODUCTION

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

Diverse physiological groups of microorganisms are present in produced water associated with corrosion of oil and gas facilities such as sulphate reducing bacteria (SRB), iron oxidizing bacteria (IOB), manganese oxidizing bacteria (MOB) nitrate reducing bacteria (NRB) and acid producing bacteria (APB) [5]. 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 microbial community in oilfield environment usually enter during drilling or application of injection water for pressure build up [6]. The genome of aerobic hydrocarbon utilizing bacteria can be determined enzymatically [7].

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 [8-10]. The description of the microbial community of an environmental sample can be done using ribosomal RNA to obtain the libraries of the cDNA of the 16S rRNA fragments [11,12]. The 16S rRNA clone libraries and sequences from the total microbial cell DNA had also be used to determine the microbial diversity in formation water from oil production wells [9,13-15]. The present study focused on the molecular characterization of acid producing bacteria from selected oilfield environments located in Niger Delta, Nigeria.

2. MATERIALS AND METHODS

2.1 Wastewater Sample Collection and Transport

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

2.2 Physicochemical Analyses

The physicochemical parameters that were analysed in the produced water samples include: Temperature, pH, electrical conductivity and total dissolved solids. Temperature and pH were determined using Thermo Scientific Orion Star A214 pH/ISE meter while the total dissolved solids and electrical conductivity were determined using YSI 3200 Conductivity Instrument [16].

2.3 Microbiological Analyses

2.3.1 Estimation of total heterotrophic bacteria in the wastewater samples

Total heterotrophic bacterial population was determined under aerobic and anaerobic conditions using the standard plate count method of enumeration. 0.1 ml dilutions of wastewater samples were aseptically inoculated into sterile plates of standard plate count agar (SPCA). A sterile glass rod (hockey stick) was used to spread the inoculum in an even pattern on

surface of agar plates in triplicates [17]. The cultured plates were incubated at 37ºC for 24 to 48 hours for aerobic culture and for seven (7) days for anaerobic culture.

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

CFU/ml = (TVC X Dilution Factor / Inoculum Volume)

2.3.2 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 10 g of peptone, 5 g of dextrose, 5 g of sodium chloride and 18 mg of phenol red powder with 1litre of distilled water. The medium was autoclaved at 121ºC for 15 minutes before use. The multiple tube fermentation technique was adopted for biocorrosion studies involving acid producing bacteria [17]. The inoculated broth was incubated at 37ºC for 7 days under aerobic and anaerobic conditions during the study [18].

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 [18].

2.3.3 Extraction and purification of acid producing bacterial DNA

The boiling method was used for extraction of 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. Thereafter, the LB broth was centrifuged at 12000rpm for 3 minutes followed by addition of 500 ul of normal saline to Ependorff tube containing cell DNA sample. The tubes were heated at 95^{o}C for 20 minutes, and then were fast cooled on ice followed by spinning at 12000rpm for 3 minutes. The cell DNA supernatant was kept at -10ºC for further procedures. After that, the extracted cell genomic DNA was quantified by Nanodrop 1000 spectrophotometer.

2.3.4 16S rRNA amplification and sequencing

The amplification was done using 16s rRNA region of rRNA gene of isolates. The primers used for amplification are 27F: 5' AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5' CGGTTACCTTGTTACGACTT-3' on an 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.

2.3.5 Sequencing

The Big Dye Terminator Kit on a 3510 ABI Sequencer was used to perform sequencing. The analysis was done by Inqaba Biotechnological, Pretoria, South Africa. The final volume of 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 sequencing and optimum conditions are 32 cycles of 96ºC for 10second, 55ºC for 5seconds and 60ºC for 4minutes.

2.4 Phylogenetic Analysis

Bioinformatics algorithm Trace edit was used to edit the sequences obtained. BLASTN was electronically used to download similar sequences from National Center for Biotechnology Information (NCBI) database. MAFFT was used to align sequences. The Neighbor-Joining method in MEGA 6.0 was adopted to infer evolutionary history of isolates [19]. The bootstrap consensus tree predicted from 500 replicates [20] was taken to represent the evolutionary history of taxa determined. The Jukes- Cantor method was used to compute evolutionary distances [21].

3. RESULTS

The results of physicochemical parameters of produced water are given in Figs. 1-4 which show graphical view of relationship of values of each parameter with sample source.

The microbiological analyses results of the produced water sample are given in Figs. 5 – 7.

3.1 Molecular Characterization of Acid Producing Bacteria from Produced Water

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

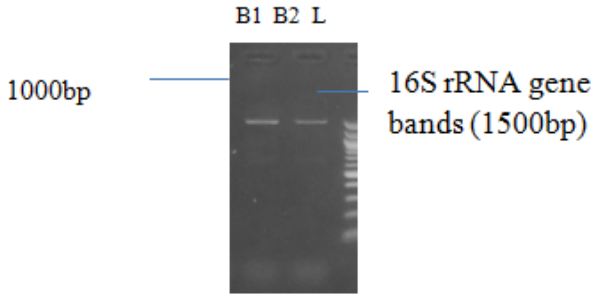

Plate 1. Agarose gel electrophoresis of the 16S rRNA gene of the study bacterial isolates. Lanes B1 and B2 represent the 16SrRNA gene bands (1500bp), lane L represents the 100bp molecular ladder

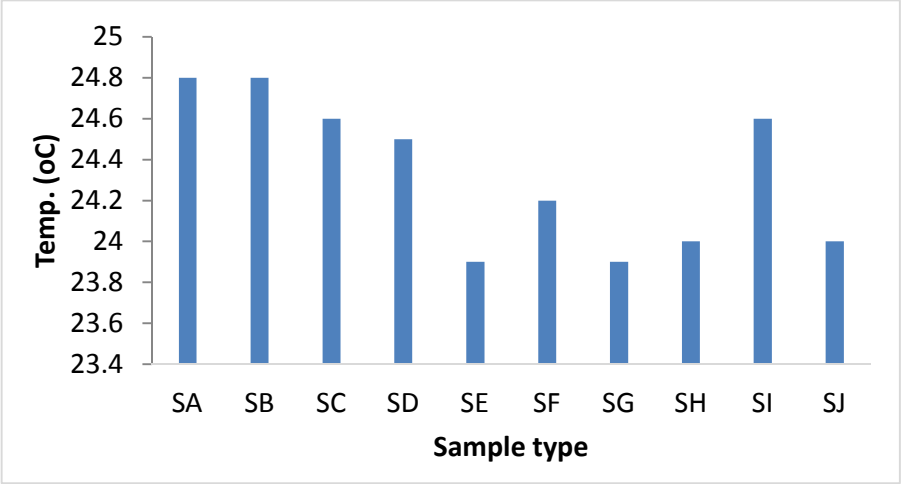

Fig. 1. The temperature values of the produced water samples
Keys: SA = Sample A; SB = Sample B; SC = Sample C…….. SJ = Sample J

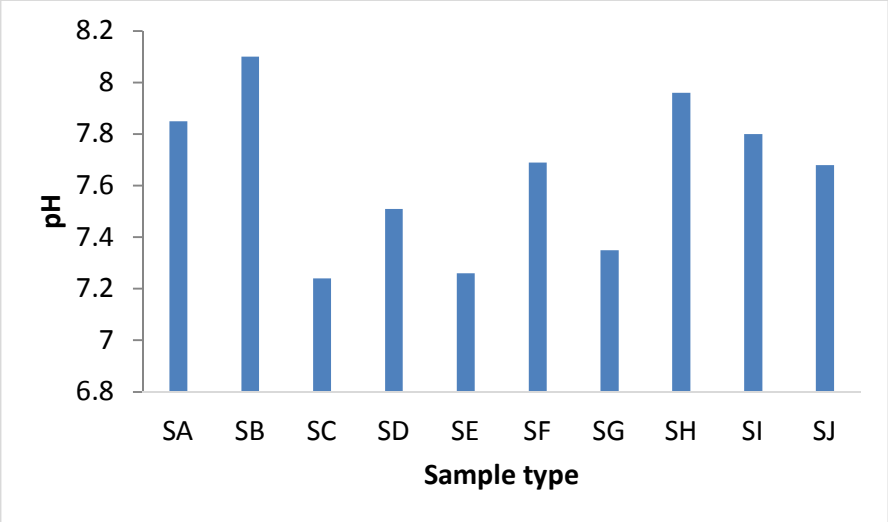

Fig. 2. The pH values of the produced water samples
Keys: Idem

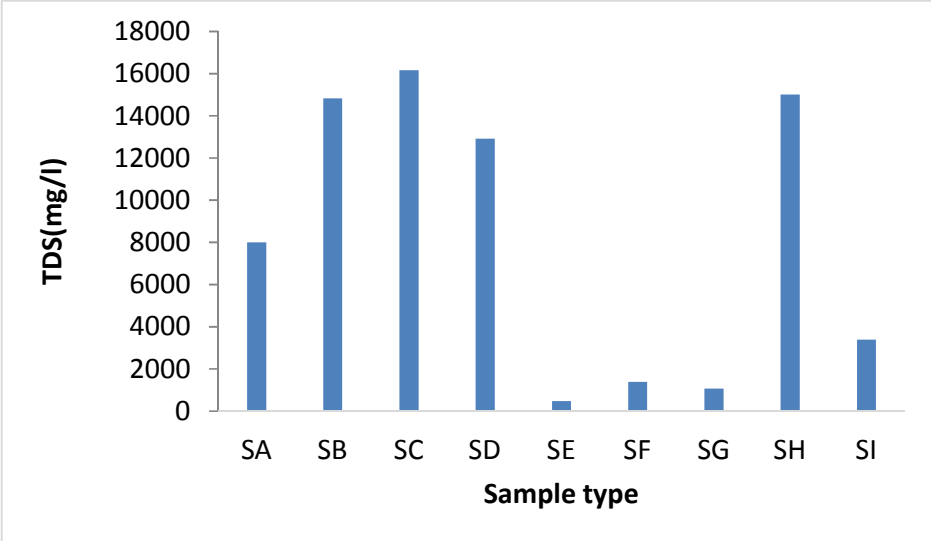

Fig. 3.The values of total dissolved solids
Keys: Idem

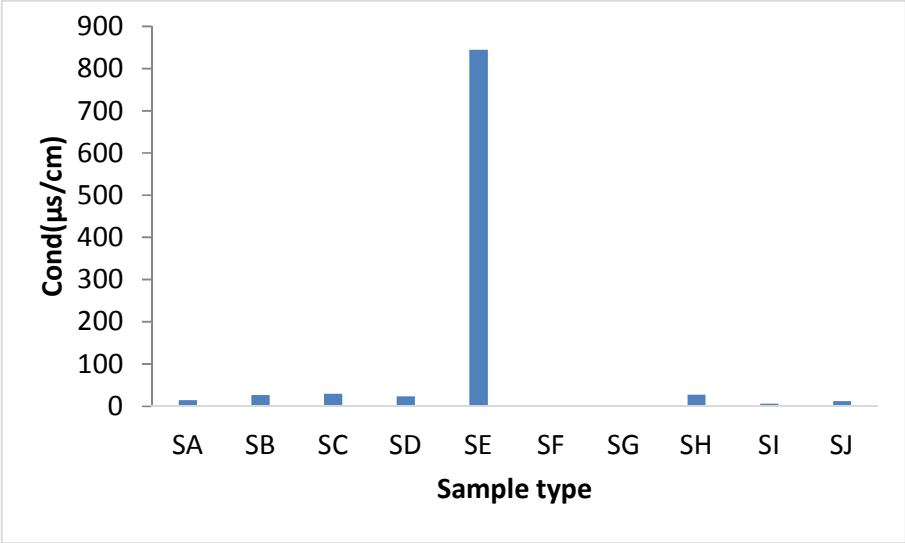

Fig. 4. The values of the electrical conductivity
Keys: Idem

4. DISCUSSION

The temperature values were highest for flow station samples (SA & SB), while that for 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 environmental temperature has on water environment where corrosive bacteria can be found as temperature changes can occur due to atmospheric conditions and seasonal variations. Awoyemi et al. [22] reported a temperature range of 26.10ºC to 26.55ºC for rainy season and 28.10^{o}C for dry season for both groundwater and surface water. This report clearly indicates that environment greatly influences changes in temperature. Onojake et al. [23] also reported that temperature of produced water from oilfield location to be within a range of 21.9^{o}C to 24.7^{o}C.

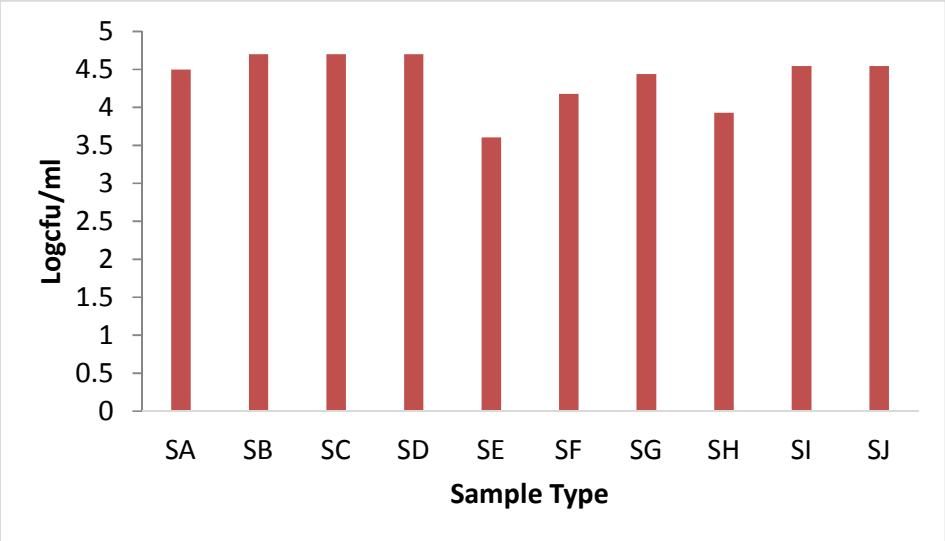

Fig. 5. Total heterotrophic bacteria population (aerobic)
Keys: Idem

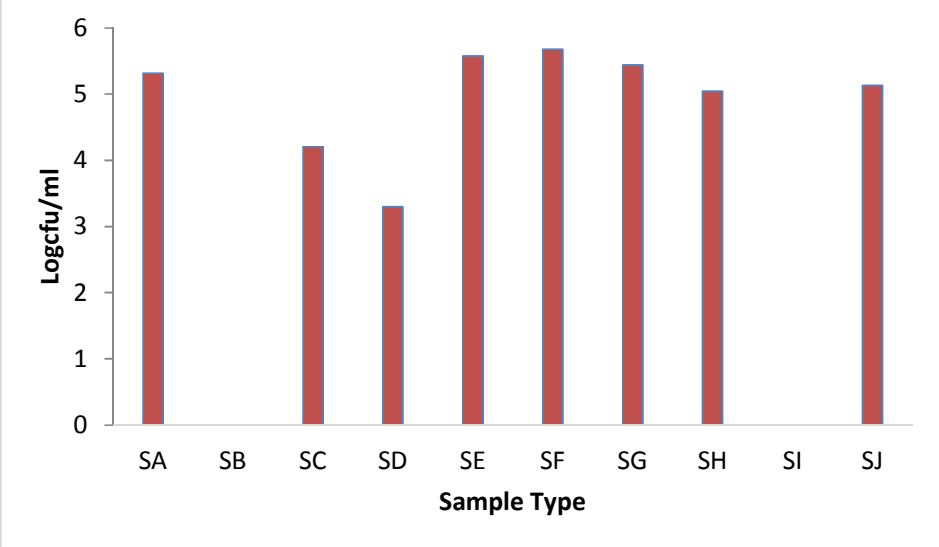

Fig. 6. Total heterotrophic bacteria population (anaerobic)
Keys: Idem

The pH values for all samples were within slightly the same 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 limit of pH (7.47 to 8.50) for inland and near shore reported by Onojake et al. [23].

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 range of (470 mg/l to 16160 mg/l, indicating greater degree of pollution by dissolved substances presence in samples. The values of 80% of samples were above the regulatory limit of 2000 mg/ml for inland area by World Health Organization [23]. Only three (3) samples had TDS values within 400 mg/ml to 1400 mg/ml. Onojake et al. [23] reported TDS values for produced water ranging from 3200 mg/ml to 7000mg/ml. TDS values also indicate greater microbial population in most of produced water sample.

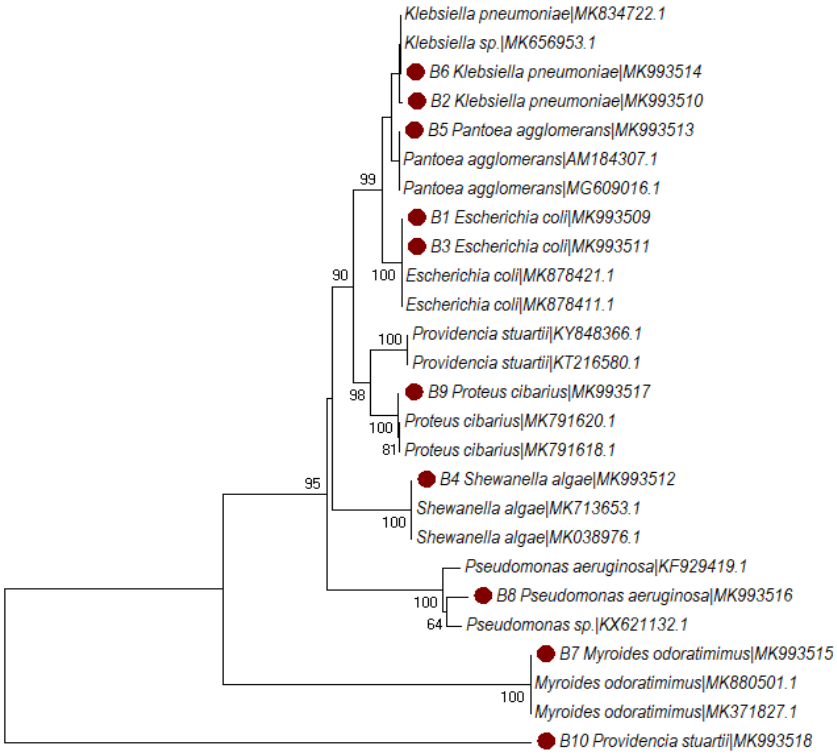

Fig. 7. Phylogenetic tree of acid producing bacteria from produced water

Electrical conductivity values show purity level of 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. [23], reported conductivity values ranging from 126.50µs/cm to 198.00µs/cm. The conductivity indicates the presence of dissolved salts and elements in the produced water samples. It is used to test purity level of water. Higher conductivity implies higher pollution rate of the produced water sample and higher microbial population degree and possibility of microbial induced corrosion.

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 period than anaerobic condition which took longer growth time. From this study results, it can be inferred that acid producing bacteria could survive in different environment and under different growth condition. This could a mode of ecological adaptation for survival strategy in certain environment. Microbial Control Specialists report [24] revealed that among 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 identified as acid producing bacteria is *Escherichia coli*, which for long was known to be 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 environment based on ability to adapt with ecological changes in different environment. It could also mean that the isolate 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 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 [17]. In all, the presence of these groups of bacteria in produced water sample also

indicates that there is obvious similarity in environment where these bacteria can be found and isolated. Acid producing bacteria like other corrosive bacteria release metabolic products which 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 solid corrosion products formation of [25,26]. *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 entire surface of metal structure. These bio-deposits act as traps and food for other corrosive microorganisms which lead to formation of a complex matrix of bacterial biofilm that further set up a corrosion potential between metal surface and 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 improve oil production [27]. More so, Biji et al. [28] 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 high cost of chemicals. It is also very interesting to note that microbial products from the acid producing bacteria (APB) are biodegradable and environmentally friendly [28].

5. CONCLUSION

The presented study about isolation and characterization of acid producing bacteria has revealed the possibility of coliform bacteria to be among the corrosive bacteria such as sulphate reducing bacteria (SRB), iron oxidizing bacteria (IOB), manganese oxidizing bacteria (MOB) etc. involved in biocorrosion of metals and industrial metallic materials. These corrosive bacteria have been and are still problematic to durability and integrity of industrial facilities today.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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